Receptor Tyrosine Kinase Regulated Growth and Remodeling of Tendon

by

Kristoffer Bradley Sugg

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular and Integrative Physiology)
in the University of Michigan
2017

Doctoral Committee:

Associate Professor Christopher L. Mendias, Chair
Professor Susan V. Brooks, Co-Chair
Professor Paul S. Cederna
Professor Eithne J. Comerford, University of Liverpool
Associate Professor Daniel E. Michele

Kristoffer Bradley Sugg

krisugg@med.umich.edu

ORCID iD: 0000-0002-0301-4083

Dedication

"In the fields of observation chance favors only the prepared mind." –Louis Pasteur

This dissertation is dedicated to my father, who taught me from a very young age the important values of hard work, dedication, loyalty, and most of all, humility.

Acknowledgements

First and foremost, I would like to thank my mentor and dear friend, Chris Mendias. I greatly appreciate your patience, guidance and enthusiasm for science. You not only taught me how to do science, but you opened my eyes to the business of science as well. Thank you for everything, but most of all, thank you for always challenging me and taking me out of my comfort zone. You set a high bar for me, but demanded nothing of me that you did not think I could handle, and because of this I turned out to be a much better scientist than I ever thought I could be. And for that I will always be grateful.

Additionally, I owe a great deal of thanks to Sue Brooks. You have been an outstanding mentor and probably give the best advice of anyone I know. Your calm demeanor always put me at ease, and your constant support was vital to my success as a graduate student. Thank you so much for always being there for me, and I appreciate the time you have invested in my development as a scientist.

I also want to thank my committee members, Dan Michele, Paul Cederna and Eithne Comerford. I appreciate you believing in me and providing valuable input that ultimately helped shape my final dissertation. Each of you have a remarkable ability of making the most complicated things seem so simple, and this helped keep me focused on the important tasks at hand. Thank you again for everything.

To the members of the Mendias Lab, thank you for accepting a guy like me with very little lab experience into your ranks and for teaching me how to function in a busy lab environment. I also want to give a special thanks to Jon Gumucio. I enjoyed completing my PhD training alongside you. You have an amazing aptitude for science and I learned so much from you.

To my UM Plastic Surgery family, thank you so much for allowing me to pursue a PhD degree. This would not have been possible without you. I will never forget your continued support throughout this process, and I cannot express enough gratitude.

To my UM MIP family, thank you for accepting me with open arms – I know I was not the typical applicant. It was great getting to work with such a talented group of individuals. I learned so much from all of you.

Lastly, I want to thank my wife, Sarah, and our four children, Makena, Kristoffer, Emma and Isla, plus one more on the way… You are the reason I get up every day and do what I do. Nothing makes me happier than coming home to you and seeing your smiling faces. You have supported me in more ways than you could ever imagine. I love you so much, and I look forward to what the future has in store for us.

Table of Contents

Dedication……ii

Acknowledgements……...iii

List of Figures……..……...vii

List of Tables……..ix

Abstract……...x

Chapter I. Introduction……..……1

Chapter II. Changes in Macrophage Phenotype and Induction of Epithelial-to-Mesenchymal Transition Genes Following Acute Achilles Tenotomy and Repair

Abstract……33

Introduction……..……...34

Methods……...36

Results……38

Discussion……...41

Acknowledgements……46

References……..…54

Chapter III. Postnatal Tendon Growth and Remodeling Requires Platelet-Derived Growth Factor Receptor Signaling

Abstract……59

Introduction……..……..60

Methods……...…62

Results……70

Discussion……...…80

Acknowledgements……90

References……105

Chapter IV. Conclusions……..110

List of Figures

1.1 Tendon stress-strain curve……23

1.2 Graphical representation of the morphological features in healthy tendon and tendinopathy……24

1.3 Overview of the major signaling pathways activated by PDGFR phosphorylation...25

2.1 Illustration of tendon repair……47

2.2 Macrophages accumulate sequentially in tendon following surgical tear and repair……48

2.3 Changes in gene expression of macrophage and neutrophil markers in the Achilles tendon following surgical tear and repair……49

2.4 Changes in gene expression of fibroblast proliferation and cell cycle control factors in the Achilles tendon following surgical tear and repair……50

2.5 Changes in gene expression of EMT-related genes in the Achilles tendon following surgical tear and repair……51

2.6 Changes in gene expression of ECM components and MMPs in the Achilles tendon following surgical tear and repair……52

3.1 Tendon fibroblasts express both PDGFRα and PDGFRβ……91

3.2 CP-673,451 inhibits phosphorylation of PDGFRα and PDGFRβ in tendon fibroblasts……92

3.3 Inhibition of PDGFR signaling prevented growth of plantaris tendons subjected to mechanical overload……93

3.4 PDGF-BB stimulates proliferation of tendon fibroblasts in vitro……94

3.5 Inhibition of PDGFR signaling decreases tendon fibroblast proliferation in vivo.....95

3.6 Microarray……96

3.7 Inhibition of PDGFR signaling prevents the expression of angiogenesis, ECM synthesis and remodeling, cell specification and proliferation, and cell migration genes in plantaris tendons subjected to mechanical overload……97

3.8 Effect of PDGF-BB treatment on collagen expression, and validation of MT1-MMP siRNA……...…98

3.9 PDGF-BB stimulates tendon fibroblast migration in vitro. (A) Schematic representation of the in vitro migration assay……99

3.10 MT1-MMP is required for PDGF-BB-mediated fibroblast migration through tendon ECM……...…100

3.11 PDGF-BB stimulation of tendon fibroblasts activates PI3K/Akt and ERK1/2 pathways, which both in turn mediate tendon fibroblast migration through tendon ECM……...…101

3.12 PDGF-BB does not regulate MT1-MMP mRNA abundance, but does increase MT1-MMP protein levels through a PI3/Akt-dependent mechanism……..…102

3.13 PDGF-BB-dependent membrane trafficking of MT1-MMP in tendon fibroblasts is regulated by the PI3K/Akt and ERK1/2 pathways……..…103

4.1 Targeting scheme for lineage tracing experiments…….....……..123

4.2 Targeting scheme for genetic inactivation of PDGFRα and PDGFRβ…...……..124

List of Tables

2.1 mRNA transcripts evaluated by quantitative PCR……..…53

3.1 Primer sequences used for quantitative PCR……...…104

Abstract

Tendon connects skeletal muscle to bone and transmits force generated from muscular contraction to produce locomotion. Despite the importance of tendon to the overall function of the musculoskeletal system, relatively little is known about the cellular and molecular mechanisms that control its growth, maintenance and repair. This lack of knowledge represents a significant barrier to improved management of tendon injuries, which remains difficult with poor outcomes. The objective of this dissertation was to determine the role of key signaling pathways activated during tendon growth and injury, and to investigate the biological mechanisms behind these responses. Since a central feature of tendon disorders is abnormal fibroblast morphology and a grossly disrupted appearance of the tendon extracellular matrix (ECM), my overall working hypothesis is molecular programs that regulate the interaction between tendon fibroblasts and their local ECM environment during tendon growth play an important role in the healing response of injured tendons. Herein, initial studies identified epithelial-to-mesenchymal transition and matrix metalloproteinases as important sets of genes upregulated in the proliferative and remodeling phases of tendon regeneration. A large number of these genes are known to be regulated by a family of receptor tyrosine kinases known as the platelet-derived growth factor receptors (PDGFRs), yet the role of PDGFR signaling in tendon growth and remodeling has remained largely unexplored. Using hindlimb

synergist ablation to overload tendons and induce tendon growth in mice, we showed that tendon fibroblasts express both PDGFRs, PDGFRα and PDGFRβ, and the inhibition of PDGFR signaling suppressed the normal growth of tendon tissue after mechanical overload due to defects in fibroblast proliferation and migration. Membrane type-1 matrix metalloproteinase (MT1-MMP) was also identified as an essential proteinase for fibroblast migration through tendon ECM. Using a combination of small molecule inhibitors and cell-surface biotinylation experiments, we showed that MT1-MMP translation was regulated by PI3K/Akt within tendon fibroblasts, while ERK1/2 controlled post-translational trafficking of MT1-MMP. Taken together, these findings demonstrate that PDGFR signaling is necessary for postnatal tendon growth and remodeling, and that MT1-MMP is a critical mediator of tendon fibroblast migration and a potential target for the treatment of tendon injuries and diseases.

Chapter I

Introduction

Background

The musculoskeletal system provides structural support, maintains form and permits movement of the body. Comprised of skeletal muscle, tendon and bone, each of these tissues display a remarkable ability in their adaptation to various physiological and pathological stimuli, which is important in determining the overall health and functional capacity of an individual. While a great deal is known about the growth processes of skeletal muscle and bone, the specific factors controlling the growth and remodeling of tendon tissue have not been clearly defined. Additionally, whereas treatment options for tendon injuries and diseases are quite limited, many therapeutic agents are currently in development for disorders of skeletal muscle and bone, and these therapies are derived from knowledge of the cellular and molecular mechanisms controlling growth of these tissues. In adult skeletal muscle, satellite cells are resident stem cells that play a critical role in muscle regeneration, and many therapies being tested for muscular dystrophies and sarcopenias target these cells (21, 67). In bone, the study of osteoclast-directed bone resorption has led to the development of bisphosphonates for the treatment of osteoporosis (11). In this regard, studies investigating the fundamental biology of

tendon growth and remodeling hold great potential for making a substantial impact on the management of tendon injuries and diseases.

Tendons are anatomically situated between force-generating muscles and the rigid support of the skeleton. Given this unique position between contractile and non-contractile tissues, tendons have the dual responsibility of transmitting force generated by muscular contractions to produce locomotion, while at the same time protecting the muscle from injury by limiting the strain placed on the muscle fibers (10). Since tendon is more compliant than bone, differences in strain concentration between these mechanically different tissues results in increased susceptibility of the tendon tissue to injury (51). Tendinopathy, a chronic degenerative process causing pain in and around tendon tissue, is a common clinical problem that accounts for 7% of all patient encounters seen in the United States on a yearly basis (68). Tendinopathies also account for 30-50% of all injuries in sports and the risk of these injuries are increasing as a larger proportion of young and old age groups participate in recreational activities (1). Additionally, organized sports programs have placed greater demands on athletic performance, requiring that athletes train longer, harder and more often, and chronic overuse has been shown to increase the risk of developing tendinopathy (65). Interestingly, while an increased level of physical activity is a strong predictor of sustaining a tendon injury, a large proportion of cases are observed in those who lead a sedentary lifestyle (41, 43, 53, 68). The quality of life for patients with tendinopathy is significantly impaired due to constant pain and loss of function. The mainstays of treatment have been to reduce inflammation and maintain mobility through physical therapy, but many patients have poor outcomes and continue to experience pain

despite treatment (68, 77). Thus, a more direct approach to the management of tendinopathies is greatly needed, and the current lack of targeted therapies is due in part to our limited mechanistic insight into the basic physiological processes controlling the growth and remodeling of tendon tissue.

The objective of this dissertation was to determine the role of key signaling pathways activated during tendon growth and injury, and to investigate the biological mechanisms behind these responses. Since a central feature of tendon disorders is abnormal fibroblast morphology and a grossly disrupted appearance of the tendon extracellular matrix (ECM), my overall working hypothesis is molecular programs that regulate the interaction between tendon fibroblasts and their local ECM environment during tendon growth play an important role in the healing response of injured tendons. In this introductory chapter, the normal structure and function of tendon are first discussed, along with a concise review of tendon mechanics. Signaling by receptor tyrosine kinases (RTKs) is then summarized, as these signaling pathways emerged from this work as critical to the regulation of tendon growth. This chapter concludes with a brief synopsis of tendinopathies and the potential to improve their management through basic science research.

Tendon Structure and Function

Tendons connect skeletal muscle to bone and transmit force generated by muscular contraction to permit movement. They are considered an extension of the skeletal muscle ECM and terminate on bone at the enthesis (10). Tendons vary in size and shape with short, thick tendons originating from the powerful muscle groups of the

proximal limb, while long, thin tendons execute fine movements of the digits (29). Smooth and efficient motion requires tendon gliding that is not restricted by adjacent tissue. For this reason, synovial sheaths form a closed system around many tendons to provide lubrication and cushion the tendon as it stretches and relaxes (10). Tendons that lack true synovial sheaths are instead surrounded by a loose, fatty and vascularized peritendinous tissue, which allows for free excursion of the tendon within its fascial compartment (29).

Tendon is a hypocellular tissue composed of elongated fibroblasts interspersed between a complex network of matrix proteins. The organizational pattern of tendon closely resembles that of skeletal muscle with distinct connective tissue layers running parallel to its longitudinal axis (29). The smallest functional unit of tendon is the collagen fibril. Collagen fibrils combine to form larger collagen fibers, and then groups of these fibers coalesce into tendon fascicles. The process of collagen fibril formation is described in more detail later. Tendon fascicles are enveloped by the endotenon, which is a delicate reticular network of connective tissue supporting a rich supply of vascular, lymphatic and neural channels (29). The tendon proper is the final structure formed by numerous tendon fascicles held together by a loose epithelial-like tissue layer called the epitenon (72). The epitenon contains larger blood vessels and nerves, and is a potential source of new tendon fibroblasts during periods of tendon growth and repair (19, 46).

During the course of embryonic development, tendons arise from a separate pool of progenitor cells than other limb tissues, including skeletal muscle, cartilage and bone (61). Tendon fibroblasts are rod- or spindle-shaped cells with long cytoplasmic extensions whose primary function is to synthesize and secrete collagen and other

matrix proteins (6). The density of fibroblasts in tendon is highest at birth and then steadily declines throughout the lifespan of an individual (42). Along similar lines, Ippolito and colleagues (26) originally described two different populations of tendon fibroblasts, namely immature proliferating tenoblasts and terminally differentiated tenocytes. As individuals approach adulthood, the number of tenoblasts decrease while terminally differentiated tenocytes become the most abundant cell population in tendon (19). The origin and identity of the stem cell population within tendon that gives rise to tenocytes is not fully understood. However, pericytes, which are a population of stem cells that exist in close proximity to the vasculature, appear to be attractive candidates (60). The remaining cells within tendon mostly consist of endothelial and smooth muscle cells of the tendon vasculature, synovial cells within the tendon sheath and chondrocytes at the enthesis (65).

The main structural protein of tendon is type I collagen, which accounts for 65-80% of the tendon dry weight (34). Type I collagen is first synthesized as a procollagen molecule within the tendon fibroblast and typically consists of two α1 and one α2 subunits. Procollagen is then secreted from the cell into the tendon ECM where its amino- and carboxy-terminal ends are cleaved to produce tropocollagen. As the final step in the collagen synthesis pathway, multiple tropocollagen molecules are covalently cross-linked resulting in the formation of the mature collagen fibril. Type I and III collagen are part of the fibrillar collagen family and play an important role in the longitudinal transmission of force during locomotion (10). Compared to type I collagen, type III collagen tends to be smaller in diameter, less organized and has decreased tensile strength (4). Type III collagen is often found at increased levels in aged tendon

or at the rupture sites of highly stressed tendons (13). Type V collagen is also a fibrillar collagen, but is present in lesser quantities and regulates the initiation of collagen fibril assembly (79). In addition to the fibrillar collagens, many other types of collagen can be found in tendon. These collagens, such as the network type IV and VI collagens, help to transmit forces laterally between cells and provide structural support for vascular and lymphatic tissue in the endotenon and epitenon (10). Mice deficient in type VI collagen display mechanically weak tendons with an increase in the number of small and aberrant collagen fibrils, indicating that type VI collagen also facilitates collagen fibril organization (27). Aside from collagen, a small but significant portion of the tendon structure in humans is formed by elastin (29). While little is known about its exact function, elastin tends to be localized to the interfascicular matrix and likely contributes to sliding between adjacent tendon fascicles and recoil after mechanical loading (63). Finally, intermixed with the collagen and non-collagen proteins of the tendon ECM is the gelatinous ground substance composed of proteoglycans (PGs) and glycosaminoglycans (GAGs). PGs and GAGs have a fixed net negative charge that allows them to retain water, which in turn helps the tendon to dissipate heat into the surrounding tissues as well as resist high compressive and tensile forces during exercise (81).

In humans, the core of the Achilles tendon will grow until the age of 17, after which protein synthesis and matrix turnover begin to decline (22). This is supported by animal studies in rodents that demonstrate tendon growth mostly occurs in an outward direction from the epitenon (19, 60). However, the lack of a discernible endotenon in rodents makes it difficult to rule out the possibility of intrafascicular growth between

tendon fascicles. Taken together, these findings suggest that the lack of tissue renewal in the core of adult tendon tissue may explain why many patients fail to improve with conservative management, and perhaps cell-based therapies that selectively utilize a fraction of cells from those connective tissue layers known to be enriched in tendon stem cells may enhance the regeneration of injured tendon tissue.

The most commonly used methods to study tendon growth in mice include treadmill running and hindlimb synergist ablation. Treadmill running is the most physiologically relevant model, and involves mice running on a treadmill for defined periods of time with progressive increases in speed, distance and incline (46). The main disadvantage of this technique is that most protocols require at least four weeks of running before any measurable changes can be observed. Additionally, motivating mice to run can be a difficult task, even when enclosed treadmills with shock grids are used (35), and constant vigilance is required from the investigator to ensure all the mice run equally for the entire duration of the protocol (17). While the hindlimb synergist ablation model is routinely used to study skeletal muscle growth (5, 45, 47), it also causes rapid and robust growth of the plantaris tendon (19). The procedure involves surgical excision of the midsubstance of the Achilles tendon to prevent the gastrocnemius and soleus muscles from plantarflexing the talocrural joint, resulting in compensatory hypertrophy of the synergist plantaris musculotendinous unit. Despite the early and reproducible results of this model, the disadvantages include the use of a supraphysiological load to induce tendon growth and the need for the investigator to have proficiency in small animal surgery.

Tendon Mechanics

The mechanical properties of tendon are determined by the macromolecular structural organization and biochemical composition of the tendon ECM (34). In general, positive adaptations of tendons to mechanical growth stimuli include increases in tendon cross-sectional area (CSA), cell density, peak stress, peak strain and type I collagen synthesis (19, 32-34, 44, 46, 80). At rest or during unloading, collagen fibrils exhibit a characteristic wavy or crimped pattern. This is the result of lateral and longitudinal stacking of tropocollagen molecules that takes place during collagen fibril assembly (78). When uniaxial tension is applied to the tendon, the stress-strain curve displays a nonlinear relationship and consists of three distinct regions (Figure 1.1). Stress is calculated as force normalized to the tendon CSA, while strain is measured as displacement normalized to the tendon resting length (44, 78). In the initial toe region of the stress-strain curve, which represents strains up to 2%, the slope is nonlinear and the crimp pattern disappears as the collagen fibrils straighten (65). Beyond this point, collagen fibrils orient themselves in the direction of the tensile force, and the tendon deforms in a linear manner due to intramolecular sliding between tropocollagen molecules (65). Strains less than 4% generally allow the tendon to return to its original length once the load is removed, but above 4% strain the collagen fibrils begin to fail (29, 65). The slope of the stress-strain curve in this linear region represents the Young's modulus, which measures the resistance of the tendon to deformation while under load, and provides useful information on the intrinsic mechanical properties of tendon (44). Finally, in the yield or failure region of the stress-strain curve, the tendon reaches its physiological limit at about 8-10% strain, which results in irreversible deformation and

visible rupture due to breakage of the intermolecular cross-links between collagen fibrils (29, 65).

Tendon is considered a viscoelastic tissue, and as such, it displays characteristics of both elastic solids and viscous fluids in response to loading and unloading. When a tendon is loaded, the extent of tissue deformation is dependent on the rate at which the load is applied (51). At low strain rates, tendons deform more and absorb more energy, while at high strain rates, tendons deform less, become stiffer and are more effective at carrying mechanical loads (78). The ground substance, which attracts and retains water due to the strong polyanionic charge of the PGs and GAGs, is one of the chief contributors to the viscoelasticity of tendon (51, 81). As tendons are stretched, the interstitial fluid is squeezed out of the tendon ECM and hydrogen bonds between the amino acid residues of collagen and water molecules are broken, which gives off energy in the form of heat (12, 51). Hysteresis refers to this loss of energy during tendon deformation, and as a result, the stress-strain curves for loading and unloading appear different (51). In situations of high-frequency cyclical stretching, certain regions of the tendon may not be able to efficiently dissipate heat into the surrounding tissues, which may cause irreversible denaturing of matrix proteins, and possibly lead to tendinopathy or acute tendon rupture (57). Lastly, two additional features that highlight the viscoelastic properties of tendon are creep and stress relaxation. Creep refers to time-dependent elongation of the tendon while under constant load, whereas stress relaxation is a time-dependent decline in the load required to maintain constant elongation of the tendon (44).

An additional factor that strongly influences the mechanical properties of tendon is the degree to which tropocollagen molecules are covalently cross-linked. As tendons are stretched, heat is generated from friction as the tropocollagen molecules slide past each other (51). One potential mechanism to reduce this fibrillar sliding, and hence the production of heat, is to increase the density of cross-links between tropocollagen molecules (51). Greater collagen cross-linking will also result in secondary changes within the tendon tissue, such as increased stiffness and a decreased amount of energy lost during loading and unloading (52). The formation of cross-links between tropocollagen molecules is a complex post-translational modification that can occur by two different methods, namely enzymatic cross-linking or non-enzymatic glycation (56). Enzymatic cross-links are mediated by lysyl oxidase, which produces highly reactive aldehyde groups on the lysine and hydroxylysine residues of tropocollagen molecules that then react with similar residues to form stable covalent bonds (54), whereas non-enzymatic cross-links are formed when lysine and hydroxylysine residues come into contact with a reducing sugar (55). The accumulation of non-enzymatic cross-links derived from the addition of glucose adducts is a signature finding in aged and diabetic tendons (55). These advanced glycation end products (AGEs) can alter the mechanical properties of tendon and lead to progressive degeneration of the tendon ECM, which increases the risk of developing tendinopathy (66). Finally, independent of the effect that AGEs have on tendon, aging in itself causes a gradual decline in the structure and function of tendon. Compared to young tendons, aged tendons typically demonstrate increased stiffness, a decreased rate of type I collagen synthesis and a decreased

content of elastin and PGs, which all contribute to an increased risk for spontaneous rupture of the tendon (28, 75).

Pathogenesis and Management of Tendinopathies

Tendinopathy is a frequent and difficult to treat clinical problem that invariably leads to a negative impact on the functional capacity of an individual. It is often considered a failure of damaged matrix proteins within the tendon to properly heal, but the exact etiology remains largely unknown. Hallmark findings of this disorder include localized pain, swelling and reduced strength and performance of the affected tendon. While frequently thought to be associated with increasing age and participation in sports, tendinopathy is routinely diagnosed in patients of all ages and levels of physical activity (1). Management of this painful condition can be particularly challenging and recovery is usually slow (65). This is especially true for athletes where higher levels of physical activity and minimal rest times make it difficult to return to sports and can lead to re-injury. Numerous treatment options exist for tendinopathy, but overall there is a lack of consensus in the literature regarding their efficacy. Indeed, some tendinopathies respond to simple interventions, while others are refractory to nearly all forms of treatment. Our insight into the pathophysiology of these diseases is constantly evolving and more sophisticated treatment strategies continue to emerge as new information is acquired through rigorous scientific pursuit.

Understanding the pathogenesis of tendinopathy has been hindered by a lack of consistent terminology. Traditionally, the term tendinitis referred to symptomatic tendons associated with chronic pain and implied an element of inflammation in its basic

etiology, especially in the initial phases of the disease process (48). Repetitive microtrauma from overload or overuse can lead to collagen fibril rupture and activation of the innate immune system (48). However, continued research has demonstrated little to no biochemical or histological evidence of inflammation in biopsies from patients with chronic tendinopathy (2). Given the uncertainty surrounding the role of inflammation in the development of tendon disorders, we prefer to use the term tendinopathy to describe an ongoing process of degeneration and failed regeneration of the tendon ECM in response to injury or disease. In clinical practice, tendinopathy refers to a spectrum of pathologies from intratendinous degenerative lesions that are a source of chronic pain, to spontaneous tendon rupture as a result of mechanical attrition.

Several features are characteristic of tendinopathy at the microscopic level (Figure 1.2). Compared to healthy tendons, tendinopathic tendons have marked disorganization and separation of collagen fibrils with a concomitant increase in mucoid ground substance (31). Collagen fibril diameter is more variable and an increased content of type III collagen contributes to mechanical weakness of the diseased tendon (13). Tendon fibroblasts adopt a rounded rather than flattened appearance and tend to be unevenly distributed throughout the tissue. Another hallmark feature of tendinopathy is neovascularization with budding capillaries invading the tendon from the paratenon (31). These capillaries are usually accompanied by the ingrowth of sensory nerve fibers that release nociceptive substances and trigger pain (59). Finally, there is absence of an inflammatory cell infiltrate in the tendon proper, although inflammation can be observed within the peritendinous tissue (31).

The management of tendinopathy can vary based on the history and extent of the condition, the severity of symptoms, and the physical demands and activity level of the patient. At the moment, conservative measures remain the mainstay of treatment for most tendinopathies. Rehabilitation, with a focus on eccentric exercises, should be a first-line therapy along with nonsteroidal anti-inflammatory drugs (NSAIDs) for short-term symptomatic relief (1). However, prolonged use of NSAIDs should be avoided due to the risk of complications and lack of proven long-term benefit. Second-line therapies include prolotherapy, platelet-rich plasma (PRP) injections and topical nitric oxide patches. As a group, the data for these are inconclusive, but their risks are low and therefore can be recommended for patients that fail first-line treatment (68). Corticosteroids should be used with caution in the treatment of tendinopathies, as the underlying cause likely does not involve inflammatory changes and their use may increase the risk of spontaneous tendon rupture (1). Nearly a third of all patients with tendinopathy fail nonoperative management (1). Surgery should only be pursued for recalcitrant cases when conservative measures have failed, or for patients with obvious operative lesions. The goal of operative management for patients with tendinopathy is to either remove the pathological areas of tendon or to induce low grade trauma to the chronically degenerated tendon ECM in an effort to restart the healing process.

A major limitation of basic science research in studying human tendinopathies is the lack of representative animal models. To date, not a single animal model exists that can fully recapitulate the biochemical and histological findings observed in diseased tendon tissue isolated from humans. The two most common techniques used to induce a tendinopathic-like condition in animals are collagenase injection and downhill treadmill

running. Collagenase injection causes proteolytic destruction of the tendon ECM (7-9, 37), but the main problem with this approach is that the collagenase enzyme is derived from the bacterium Clostridium histolyticum. Recognized as a foreign material by the body, the enzyme causes a local inflammatory reaction in addition to proteolysis that is uncharacteristic of tendinopathies. In contrast to the collagenase model of tendinopathy, downhill treadmill running invokes tendinopathy through a change in the mechanical environment rather than through use of a chemical agent. Developed by Soslowsky and colleagues (69), rats are subjected to a running protocol that equates to a total daily distance of 1 km and occurs at a 10° decline. While this model causes less inflammation than the collagenase injection, the damage that occurs to the supraspinatus tendon likely results from impingement of the tendon on the acromion rather than a true tendinopathy that develops from chronic overuse. Finally, there are presently no transgenic models of human tendinopathy, owing to the lack of cellular and molecular targets for gene inactivation. Improved animal models of human tendinopathy are greatly needed to help elucidate the basic mechanisms that underlie chronic degeneration of the tendon ECM, and to facilitate the development of better prevention and treatment strategies for this complex disease process.

Receptor Tyrosine Kinase Signaling

RTKs are an evolutionarily conserved family of high-affinity cell surface receptors that upon growth factor binding activate a complex network of intracellular signaling pathways. In humans, there are 58 known RTKs, which can be grouped into 20 subfamilies based upon the sequence homology of their cognate ligands (40). While

there are slight differences in the structural mechanisms by which ligands induce RTK oligomerization and activation, most of this review will focus its discussion of RTK signaling on the platelet-derived growth factor receptors α (PDGFRα) and β (PDGFRβ), since these receptors are targeted in Chapter III. PDGFRs play an important role in many developmental programs, primarily within mesenchymal tissues, due to their ability to regulate a diverse set of cellular responses, including proliferation, differentiation, cell survival, metabolism, cell migration and cell-cycle control (40, 76). Additionally, dysfunctional PDGFR activity has been implicated in a myriad of diseases such as cancer, diabetes, inflammation and atherosclerosis (3, 40). Although PDGFRs are essential components of complex signaling networks that are active during embryonic development and in numerous pathological conditions, there is limited evidence to support the role of PDGFR signaling in normal physiological functions, such as tissue growth, in the adult (3).

In general, all RTKs share a similar structure that consists of an extracellular ligand-binding domain at the N terminus, a single-pass hydrophobic transmembrane domain and an intracellular conserved tyrosine kinase domain (TKD) at the C terminus (40, 76). The extracellular region tends to be of variable length and composition, while the intracellular region is the most conserved, and sometimes contains a TKD that is separated into two parts by a short sequence known as the kinase insert domain (KID), which is typical of the PDGFRs (40). The PDGFRs are present at the cell membrane as monomers, and have an extracellular region that consists of five immunoglobulin-like repeats (3, 58). In the absence of ligand binding, the catalytic site of the kinase is sterically blocked by an activation loop, which is an important regulatory element found

in the TKD of the PDGFRs (50). This state of suppressed PDGFR kinase activity is referred to as *cis*-autoinhibition, and is further stabilized by sequences within the juxtamembrane region that come into contact with the activation loop (40). Upon ligand binding and receptor dimerization, key tyrosine residues in the juxtamembrane region and activation loop are autophosphorylated in *trans*, which destabilizes the autoinhibitory interactions and induces a conformational change that promotes PDGFR activation (25, 40). Signaling and adaptor proteins can then recognize these and other phosphotyrosine residues, and bind to them in a sequence-specific manner through the use of Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains for signal propagation (3).

Both PDGFRα and PDGFRβ activate multiple downstream signaling pathways that control key biological functions in many different cell types. The expression patterns of the PDGFRs can be complex, but PDGFRα is mainly expressed by mesenchymal cells and PDGFRβ has particularly strong expression in the vasculature and by perivascular cells (3). While PDGFR phosphorylation is known to recruit at least 10 SH2 domain-containing proteins, a dominant signaling pathway has yet to be identified for either receptor subtype (23, 24). However, the prevailing concept at the moment is that PDGFRs activate redundant signaling pathways that control transcription of a common set of genes such that the role of any individual signaling pathway is less important than their additive effect on the transmitted signal (14, 39). Nonetheless, the major signaling pathways activated by PDGFR phosphorylation are the Ras/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (Figure 1.3). It is also important to note that these signaling pathways are tightly regulated by several

mechanisms that either attenuate or terminate the transmitted signal in order to prevent PDGFR dysfunction, including antagonistic ligands, tyrosine phosphatases and ligand-induced receptor internalization and degradation (58).

The Ras/MAPK pathway is highly conserved and plays pivotal roles in the control of proliferation, differentiation and cell migration (36). Upon PDGFR phosphorylation, the adaptor protein growth factor receptor-bound protein 2 (Grb2) binds to specific phosphotyrosine residues on the receptor through its SH2 domain and then recruits the guanine nucleotide exchange factor son of sevenless (Sos) to the membrane through its SH3 domain (58). The Grb2/Sos complex promotes the exchange of GDP for GTP on the small G protein Ras (58). Once in the active GTP-bound state, Ras first activates Raf, which in turn phosphorylates the S217 and S221 sites in the activation loop of mitogen-activated protein kinase kinases 1 and 2 (MEK1/2) (36). Activated MEK1/2 then phosphorylates the T202 and Y204 sites in the activation loop of extracellular signal-related kinases 1 and 2 (ERK1/2) (36). Raf and ERK1/2 are serine/threonine protein kinases, while MEK1/2 is a serine/tyrosine/threonine protein kinase (36). As the final step in the Ras/MAPK pathway, activated ERK1/2 is translocated to the nucleus where it regulates the activities of several transcription factors via phosphorylation (58).

Activation of the PI3K pathway by PDGFRs promotes proliferation, cell survival, cell migration and cell growth (3). PI3K is a heterodimer that consists of the regulatory subunit p85, which contains two SH2 and one SH3 domain, and the catalytic subunit p110 (58). Upon PDGFR phosphorylation, PI3K binds to specific phosphotyrosine residues on the receptor and is responsible for the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to the second messenger phosphatidylinositol 3,4,5-

trisphosphate (PIP_3) (40). In contrast, phosphatase and tensin homolog (PTEN) catalyzes the dephosphorylation of PIP_3 to PIP_2, and as such, is a negative regulator of the PI3K pathway (58). Protein kinase B (Akt) is a serine/threonine protein kinase that binds PIP_3 at the membrane through its N-terminal pleckstrin homology (PH) domain and is activated by phosphoinositide-dependent kinase-1 (PDK1) via phosphorylation at the T308 site in its activation loop (18). An additional S473 site on Akt can be phosphorylated by the mechanistic target of rapamycin complex 2 (mTORC2), and phosphorylation of both sites is required for full activation of Akt (38). Following its phosphorylation by PDK1, Akt is released into the cytosol where it acts on mTORC1 by inhibiting tuberous sclerosis proteins 1 and 2 (TSC1/2) (18). TSC2 contains a GTPase activating protein (GAP) domain that normally promotes the exchange of GTP for GDP on the small G protein Ras homolog enriched in brain (Rheb), but inhibition of TSC2 by Akt favors the active GTP-bound state of Rheb, and hence functions as an activator of mTORC1 (82). TSC1 does not have a GAP domain, but instead stabilizes TSC2 and likely prevents it from degradation (82). mTORC1 is a serine/threonine protein kinase that signals downstream through multiple signaling proteins including p70 S6 kinase (p70S6K) via phosphorylation at the T389 site in its linker domain (71). Additional T421 and S424 sites in the pseudosubstrate domain of p70S6K can be phosphorylated by various upstream kinases, which lead to conformational changes that enhance the activation of p70S6K (71). Activated p70S6K increases protein synthesis by stimulating ribosomal protein S6 (rpS6) and eukaryotic translation initiation factor 4B (eIF4B), factors that increase ribosomal biogenesis and initiate mRNA translation, respectively (82). Finally, phosphorylation of eukaryotic translation initiation factor 4E-binding protein

1 (4EBP1) by mTORC1 releases its inhibition on eIF4E, which is bound to the 5' cap structure of mRNA, and allows mRNA translation to proceed (82).

In both mice and humans, PDGFs function as secreted hetero- or homodimers of disulfide-linked polypeptide chains (PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and PDGF-AB) (3, 23, 24). All PDGFs contain a core domain with a conserved set of eight cysteine residues, which have been shown to be necessary and sufficient for receptor binding and activation (3). The PDGF-AB heterodimer can be found in human platelets, and while its physiological importance is still under debate, homodimers appear to be dominant form of PDGF ligands in vivo (3). Based on cell culture experiments, multiple PDGF-PDGFR interactions have been described in vitro, but functional evidence suggests the actual number of PDGF-PDGFR interactions in vivo is more limited. PDGF-AA and PDGF-CC bind to PDGFR$\alpha\alpha$, while PDGF-BB and PDGF-DD have a higher affinity for PDGFR$\beta\beta$ (24). Differences in sequence structure and proteolytic processing between the PDGF ligands helps distinguish them from one another. The N-terminal prodomains of PDGF-AA and PDGF-BB are removed intracellularly by furin convertases and each are secreted in its active form (15), while PDGF-CC and PDGF-DD are secreted in their latent forms and require activation outside of the cell (15). Activation of PDGF-CC and PDGF-DD requires cleavage of their N-terminal complement subcomponents C1r/C1s, sea urchin uEGF and human BMP-1 (CUB) domains (24). The endogenous proteases responsible for the dissociation of the CUB domains from PDGF-CC and PDGF-DD have not been identified in vivo, although plasmin and tissue plasminogen activator (tPA) have been shown to accomplish this task in cell culture (16). Following secretion from the cell, PDGF-AA and PDGF-BB can

establish diffusion gradients in the interstitium by binding to various ECM components such as matrix proteoglycans due to their C-terminal retention motifs that contain a stretch of positively charged basic amino acid residues (3, 30). These motifs help retain the PDGF ligands in the ECM near the cells that produce them until they are released by paracrine factors such as thrombin (24, 70). Although PDGF-CC and PDGF-DD lack retention motifs, their CUB domains may regulate the extracellular distribution of these ligands through protein-protein interactions (3). With regard to tendon, the role of PDGFR signaling in tendon growth and injury has remained largely unexplored. While PDGFRα and PDGFRβ are mainly expressed by mesenchymal and perivascular cells, respectively, the expression pattern of PDGFRs by tendon fibroblasts is not known (3). In the case of tendon growth, it remains possible that the local bioavailability of PDGF increases by mechanical or proteolytic activation of latent PDGF in the tendon ECM similar to transforming growth factor beta (TGF-β) (20). However, in the event of tendon injury, PDGF secretion by infiltrating platelets and other inflammatory cells such as macrophages likely accounts for the paracrine effects of PDGF on tendon tissue (49).

Dissertation Aims

The main focus of this dissertation is to address a fundamental question in musculoskeletal biology – how do adult tendons grow? In response to physiological loading, adult tendon tissue increases in size and undergoes changes to its matrix composition, which ultimately leads to an increase in athletic performance. However, if loading is too much or too frequent, patients may develop tendinopathy as a result of cumulative damage to the tendon ECM. Given the relationship between tendon growth

and injury, insights gained from studies of the tendon growth are likely to facilitate a better understanding of tendon healing following injury. Therefore, the objective of this dissertation was to determine the role of key signaling pathways activated during tendon growth and injury, and to investigate the biological mechanisms behind these responses. Since a central feature of tendon disorders is abnormal fibroblast morphology and a grossly disrupted appearance of the tendon ECM, my overall working hypothesis is molecular programs that regulate the interaction between tendon fibroblasts and their local ECM environment during tendon growth play an important role in the healing response of injured tendons. In Chapter II, transection of the Achilles tendon followed by acute repair is accompanied by significant changes in the tendon transcriptome, including upregulation of numerous matrix metalloproteinase (MMP) and epithelial-to-mesenchymal transition (EMT) genes, many of which are known to be regulated by PDGFR signaling. PDGFs have been investigated as a potential form of treatment in translational studies to enhance tendon healing following injury. They have been used with modest success in canine models of intrasynovial flexor tendon repair (73, 74), and their use improved mechanical properties in a rat model of Achilles tendinopathy induced by collagenase injection compared to other common treatments, including platelet-rich plasma and steroids (64). However, most of the previous work on PDGF function in tendon is correlative. This dissertation makes a substantial contribution to the literature by testing the importance of PDGFR signaling in the context of tendon growth with loss of function experiments. In Chapter III, the inhibition of PDGFR signaling suppressed the normal growth of tendon tissue after mechanical overload due to defects in fibroblast proliferation and migration, and membrane type-1

matrix metalloproteinase (MT1-MMP) was identified as an essential proteinase for fibroblast migration through tendon ECM. Taken together, these findings demonstrate that PDGFR signaling is necessary for postnatal tendon growth and remodeling, and that MT1-MMP is a critical mediator of tendon fibroblast migration and a potential target for the treatment of tendon injuries and diseases.

Acknowledgements

This work was supported by NIH grants R01-AR063649, F31-AR065931 and F32-AR067086. Portions of this chapter were submitted for publication: Mead MP, Gumucio JP, Awan TM, Mendias CL, Sugg KB. Pathogenesis and management of tendinopathies in sports medicine. *J Sci Med Sport*. 2017. Under review.

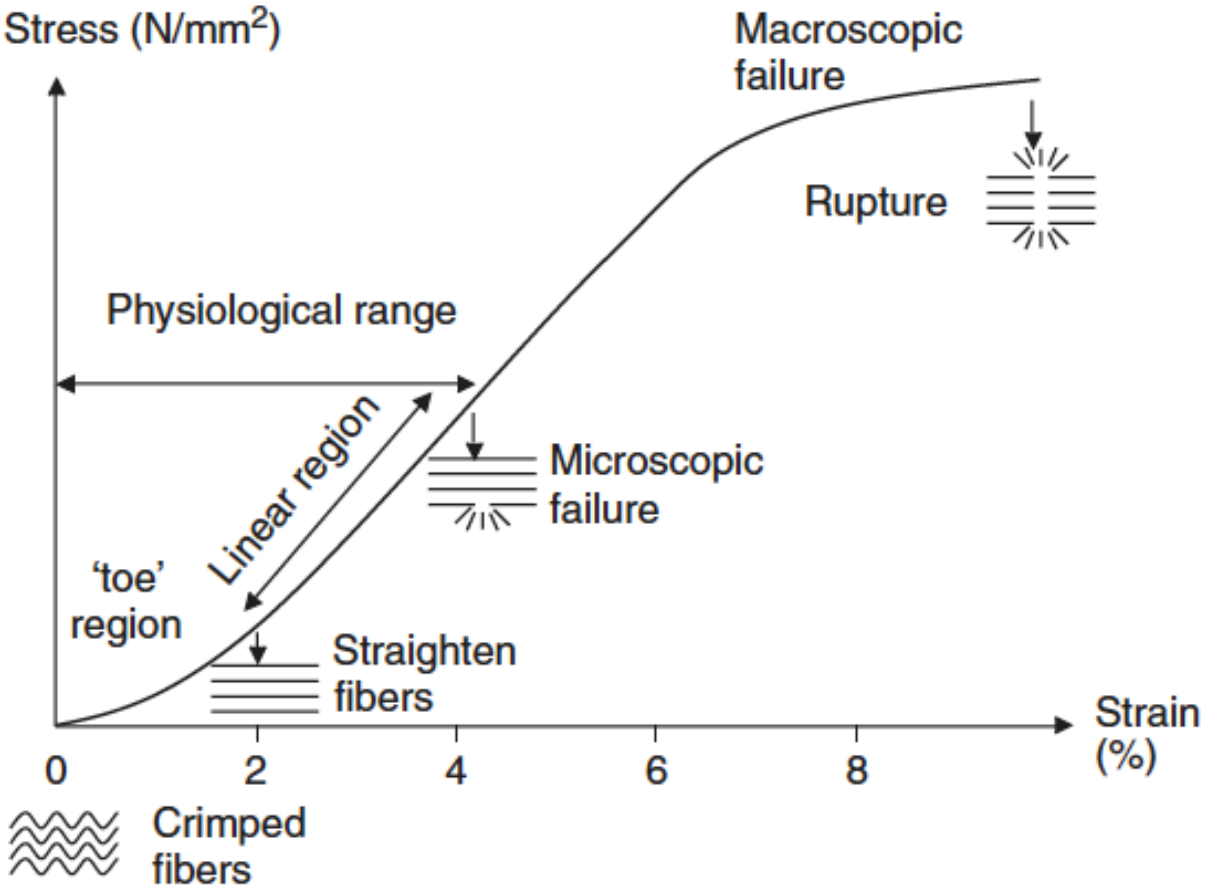

Figure 1.1. Tendon stress-strain curve (78).

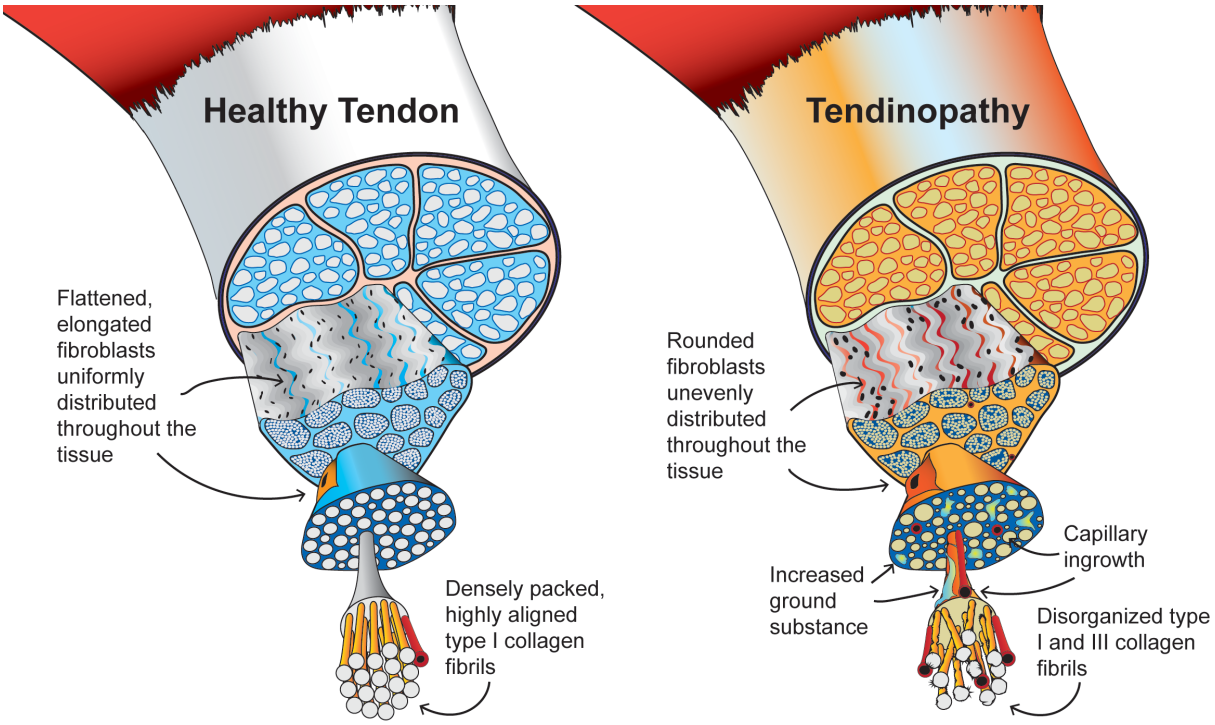

Figure 1.2. Graphical representation of the morphological features in healthy tendon and tendinopathy. Modified from Scott et al (62).

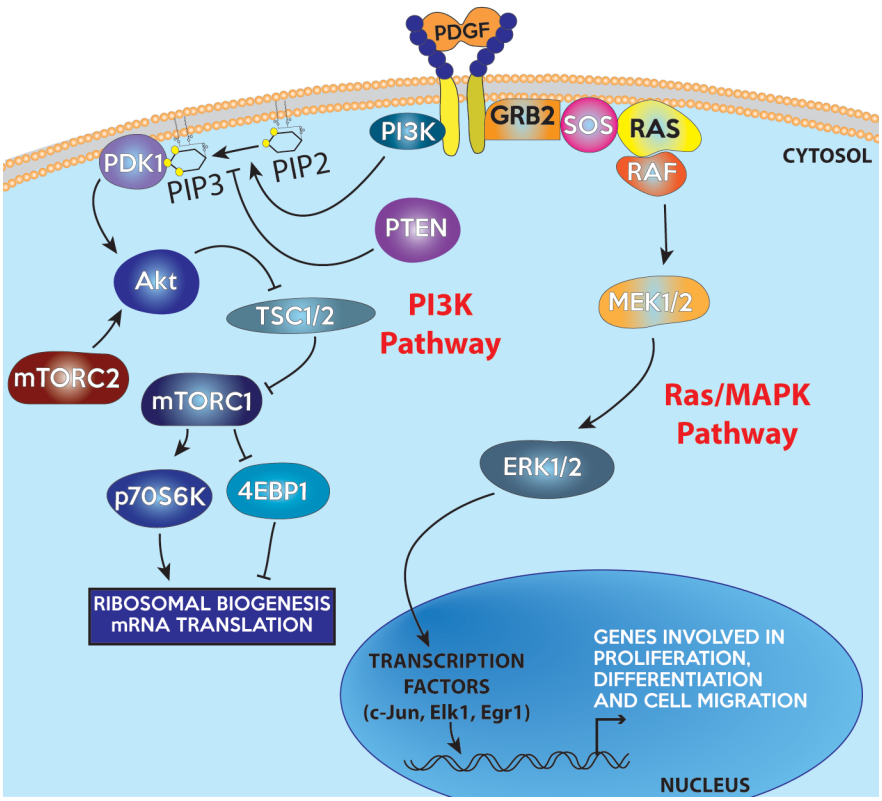

Figure 1.3. Overview of the major signaling pathways activated by PDGFR phosphorylation. Modified from Gumucio et al (18).

References

1. Ackermann PW, Renström P. Tendinopathy in sport. *Sports Health* 4: 193–201, 2012.

2. Alfredson H, Forsgren S, Thorsen K, Lorentzon R. In vivo microdialysis and immunohistochemical analyses of tendon tissue demonstrated high amounts of free glutamate and glutamate NMDAR1 receptors, but no signs of inflammation, in Jumper's knee. *J Orthop Res* 19: 881–886, 2001.

3. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes & Development* 22: 1276–1312, 2008.

4. Birk DE, Mayne R. Localization of collagen types I, III and V during tendon development. Changes in collagen types I and III are correlated with changes in fibril diameter. *European Journal of Cell Biology* 72: 352–361, 1997.

5. Calve S, Isaac J, Gumucio JP, Mendias CL. Hyaluronic acid, HAS1, and HAS2 are significantly upregulated during muscle hypertrophy. *AJP: Cell Physiology* 303: C577–C588, 2012.

6. Canty EG, Lu Y, Meadows RS, Shaw MK, Holmes DF, Kadler KE. Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. *The Journal of Cell Biology* 165: 553–563, 2004.

7. Dahlgren LA, Mohammed HO, Nixon AJ. Expression of insulin-like growth factor binding proteins in healing tendon lesions. *J Orthop Res* 24: 183–192, 2006.

8. Dahlgren LA, Mohammed HO, Nixon AJ. Temporal expression of growth factors and matrix molecules in healing tendon lesions. *J Orthop Res* 23: 84–92, 2006.

9. Dahlgren LA, van der Meulen MCH, Bertram JEA, Starrak GS, Nixon AJ. Insulin-like growth factor-I improves cellular and molecular aspects of healing in a collagenase-induced model of flexor tendinitis. *J Orthop Res* 20: 910–919, 2002.

10. Davis ME, Gumucio JP, Sugg KB, Bedi A, Mendias CL. MMP inhibition as a potential method to augment the healing of skeletal muscle and tendon extracellular matrix. *J Appl Physiol* 115: 884–891, 2013.

11. Drake MT, Clarke BL, Khosla S. Bisphosphonates: mechanism of action and role in clinical practice. *Mayo Clin Proc* 83: 1032–1045, 2008.

12. Elliott DM, Robinson PS, Gimbel JA, Sarver JJ, Abboud JA, Iozzo RV, Soslowsky LJ. Effect of Altered Matrix Proteins on Quasilinear Viscoelastic Properties in Transgenic Mouse Tail Tendons. *Ann Biomed Eng* 31: 599–605, 2003.

13. Eriksen HA, Pajala A, Leppilahti J, Risteli J. Increased content of type III collagen at the rupture site of human Achilles tendon. *J Orthop Res* 20: 1352–1357, 2002.

14. Fambrough D, McClure K, Kazlauskas A, Lander ES. Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. *Cell* 97: 727–741, 1999.

15. Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine & Growth Factor Reviews* 15: 197–204, 2004.

16. Fredriksson L, Li H, Fieber C, Li X, Eriksson U. Tissue plasminogen activator is a potent activator of PDGF-CC. *The EMBO Journal* 23: 3793–3802, 2004.

17. Ghosh S, Golbidi S, Werner I, Verchere BC, Laher I. Selecting exercise regimens and strains to modify obesity and diabetes in rodents: an overview. *Clin Sci* 119: 57–74, 2010.

18. Gumucio JP, Mendias CL. Atrogin-1, MuRF-1, and sarcopenia. *Endocrine* 43: 12–21, 2012.

19. Gumucio JP, Phan AC, Ruehlmann DG, Noah AC, Mendias CL. Synergist ablation induces rapid tendon growth through the synthesis of a neotendon matrix. *J Appl Physiol* 117: 1287–1291, 2014.

20. Gumucio JP, Sugg KB, Mendias CL. TGF-β Superfamily Signaling in Muscle and Tendon Adaptation to Resistance Exercise. *Exercise and Sport Sciences Reviews* 43: 93–99, 2015.

21. Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* 91: 534–551, 2001.

22. Heinemeier KM, Schjerling P, Heinemeier J, Magnusson SP, KJAeR M. Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb (14)C. *The FASEB Journal* 27: 2074–2079, 2013.

23. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiological Reviews* 79: 1283–1316, 1999.

24. Hoch RV. Roles of PDGF in animal development. *Development* 130: 4769–4784, 2003.

25. Hubbard SR, Miller WT. Receptor tyrosine kinases: mechanisms of activation and signaling. *Current Opinion in Cell Biology* 19: 117–123, 2007.

26. Ippolito E, Natali PG, Postacchini F, Accinni L, De Martino C. Morphological, immunochemical, and biochemical study of rabbit achilles tendon at various ages. *J Bone Joint Surg Am* 62: 583–598, 1980.

27. Izu Y, Ansorge HL, Zhang G, Soslowsky LJ, Bonaldo P, Chu M-L, Birk DE. Dysfunctional tendon collagen fibrillogenesis in collagen VI null mice. *Matrix Biology* 30: 53–61, 2011.

28. Kannus P, Jozsa L. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. *J Bone Joint Surg Am* 73: 1507–1525, 1991.

29. Kannus P. Structure of the tendon connective tissue. *Scandinavian Journal of Medicine & Science in Sports* 10: 312–320, 2000.

30. Kelly JL, Sánchez A, Brown GS, Chesterman CN, Sleigh MJ. Accumulation of PDGF B and cell-binding forms of PDGF A in the extracellular matrix. *The Journal of Cell Biology* 121: 1153–1163, 1993.

31. Khan KM, Cook JL, Bonar F, Harcourt P, Astrom M. Histopathology of common tendinopathies. Update and implications for clinical management. *Sports Med* 27: 393–408, 1999.

32. KJAeR M, Langberg H, Heinemeier K, Bayer ML, Hansen M, Holm L, Doessing S, Kongsgaard M, Krogsgaard MR, Magnusson SP. From mechanical loading to collagen synthesis, structural changes and function in human tendon. *Scandinavian Journal of Medicine & Science in Sports* 19: 500–510, 2009.

33. Kjaer M, Magnusson P, Krogsgaard M, Moller JB, Olesen J, Heinemeier K, Hansen M, Haraldsson B, Koskinen S, Esmarck B, Langberg H. Extracellular matrix adaptation of tendon and skeletal muscle to exercise. *J Anat* 208: 445–450, 2006.

34. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiological Reviews* 84: 649–698, 2004.

35. Knab AM, Bowen RS, Moore-Harrison T, Hamilton AT, Turner MJ, Lightfoot JT. Repeatability of exercise behaviors in mice. *Physiology & Behavior* 98: 433–440, 2009.

36. Kyriakis JM, Avruch J. Mammalian MAPK Signal Transduction Pathways Activated by Stress and Inflammation: A 10-Year Update. *Physiological Reviews* 92: 689–737, 2012.

37. Lake SP, Ansorge HL, Soslowsky LJ. Animal models of tendinopathy. *Disabil Rehabil* 30: 1530–1541, 2009.

38. Laplante M, Sabatini DM. mTOR signaling at a glance. *Journal of Cell Science* 122: 3589–3594, 2009.

39. Lehti K, Allen E, Birkedal-Hansen H, Holmbeck K, Miyake Y, Chun T-H, Weiss SJ. An MT1-MMP-PDGF receptor-beta axis regulates mural cell investment of the microvasculature. *Genes & Development* 19: 979–991, 2005.

40. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 141: 1117–1134, 2010.

41. Lian OB. Prevalence of Jumper's Knee Among Elite Athletes From Different Sports: A Cross-sectional Study. *Am J Sports Med* 33: 561–567, 2005.

42. M J Moore ADB. A quantitative ultrastructural study of rat tendon from birth to maturity. *J Anat* 153: 163–169, 1987.

43. Magnussen RA, Dunn WR, Thomson AB. Nonoperative treatment of midportion Achilles tendinopathy: a systematic review. *Clin J Sport Med* 19: 54–64, 2009.

44. Magnusson SP, Hansen P, KJAeR M. Tendon properties in relation to muscular activity and physical training. *Scandinavian Journal of Medicine & Science in Sports* 13: 211–223, 2003.

45. McCarthy JJ, Mula J, Miyazaki M, Erfani R, Garrison K, Farooqui AB, Srikuea R, Lawson BA, Grimes B, Keller C, Van Zant G, Campbell KS, Esser KA, Dupont-Versteegden EE, Peterson CA. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development* 138: 3657–3666, 2011.

46. Mendias CL, Gumucio JP, Bakhurin KI, Lynch EB, Brooks SV. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. *J Orthop Res* 30: 606–612, 2011.

47. Mendias CL, Schwartz AJ, Grekin JA, Gumucio JP, Sugg KB. Changes in muscle fiber contractility and extracellular matrix production during skeletal muscle hypertrophy. *J Appl Physiol* 122: 571–579, 2017.

48. Millar NL, Hueber AJ, Reilly JH, Xu Y, Fazzi UG, Murrell GAC, McInnes IB. Inflammation Is Present in Early Human Tendinopathy. *Am J Sports Med* 38: 2085–2091, 2010.

49. Molloy T, Wang Y, Murrell G. The roles of growth factors in tendon and ligament healing. *Sports Med* 33: 381–394, 2003.

50. Nolen B, Taylor S, Ghosh G. Regulation of Protein Kinases. *Molecular Cell* 15: 661–675, 2004.

51. Paxton JZ, Baar K. Tendon mechanics: the argument heats up. *Journal of Applied Physiology* 103: 423–424, 2007.

52. Reddy GK, Stehno-Bittel L, Enwemeka CS. Glycation-Induced Matrix Stability in the Rabbit Achilles Tendon. *Archives of Biochemistry and Biophysics* 399: 174–180, 2002.

53. Rees JD. Current concepts in the management of tendon disorders. *Rheumatology (Oxford)* 45: 508–521, 2006.

54. Reiser K, McCormick RJ, Rucker RB. Enzymatic and nonenzymatic cross-linking of collagen and elastin. *The FASEB Journal* 6: 2439–2449, 1992.

55. Reiser KM. Nonenzymatic glycation of collagen in aging and diabetes. *Experimental Biology and Medicine* 196: 17–29, 1991.

56. Reiser KM. Influence of age and long-term dietary restriction on enzymatically mediated crosslinks and nonenzymatic glycation of collagen in mice. *J Gerontol* 49: B71–9, 1994.

57. Rich JCP-KDLBT, Becker DL, Rich T. The Pathogenesis of Tendon Microdamage in Athletes: the Horse as a Natural Model for Basic Cellular Research. *Journal of Comparative Pathology* 147: 227–247, 2012.

58. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 103: 211–225, 2000.

59. Schubert TEO. Achilles tendinosis is associated with sprouting of substance P positive nerve fibres. *Ann Rheum Dis* 64: 1083–1086, 2005.

60. Schwartz AJ, Sarver DC, Sugg KB, Dzierzawski JT, Gumucio JP, Mendias CL. p38 MAPK signaling in postnatal tendon growth and remodeling. *PLoS ONE* 10: e0120044, 2015.

61. Schweitzer R, Zelzer E, Volk T. Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates. *Development* 137: 2807–2817, 2010.

62. Scott A, Huisman E, Khan K. Conservative treatment of chronic Achilles tendinopathy. *CMAJ* 183: 1159–1165, 2011.

63. Screen HRC, Berk DE, Kadler KE, Ramirez F, Young MF. Tendon Functional Extracellular Matrix. *J Orthop Res* 33: 793–799, 2015.

64. Shah V, Bendele A, Dines JS, Kestler HK, Hollinger JO, Chahine NO, Hee CK. Dose-response effect of an intra-tendon application of recombinant human platelet-derived growth factor-BB (rhPDGF-BB) in a rat Achilles tendinopathy model. *J Orthop Res* 31: 413–420, 2012.

65. Sharma PP, Maffulli NN. Tendon injury and tendinopathy: healing and repair. *J Bone Joint Surg Am* 87: 187–202, 2005.

66. Shi L, Rui YF, Li G, Wang C. Alterations of tendons in diabetes mellitus: what are the current findings? *International Orthopaedics (SICOT)* 39: 1465–1473, 2015.

67. Shi X. Muscle stem cells in development, regeneration, and disease. *Genes & Development* 20: 1692–1708, 2006.

68. Skjong CC, Meininger AK, Ho SSW. Tendinopathy Treatment: Where is the Evidence? *CSM* 31: 329–350, 2012.

69. Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, Carpenter JE. Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. *Journal of Shoulder and Elbow Surgery* 9: 79–84, 2000.

70. Soyombo AA, DiCorleto PE. Stable expression of human platelet-derived growth factor B chain by bovine aortic endothelial cells. Matrix association and selective proteolytic cleavage by thrombin. *J Biol Chem*.

71. Tavares MR, Pavan ICB, Amaral CL, Meneguello L, Luchessi AD, Simabuco FM. The S6K protein family in health and disease. *Life Sciences* 131: 1–10, 2015.

72. Taylor SH, Al-Youha S, Van Agtmael T, Lu Y, Wong J, McGrouther DA, Kadler KE. Tendon Is Covered by a Basement Membrane Epithelium That Is Required for Cell Retention and the Prevention of Adhesion Formation. *PLoS ONE* 6: e16337, 2011.

73. Thomopoulos S, Das R, Sakiyama-Elbert S, Silva MJ, Charlton N, Gelberman RH. bFGF and PDGF-BB for Tendon Repair: Controlled Release and Biologic Activity by Tendon Fibroblasts In Vitro. *Ann Biomed Eng* 38: 225–234, 2009.

74. Thomopoulos S, Zaegel M, Das R, Harwood FL, Silva MJ, Amiel D, Sakiyama-Elbert S, Gelberman RH. PDGF-BB released in tendon repair using a novel delivery system promotes cell proliferation and collagen remodeling. *J Orthop Res* 25: 1358–1368, 2007.

75. Tuite DJ, Renström PAFH, O'Brien M. The aging tendon. *Scandinavian Journal of Medicine & Science in Sports* 7: 72–77, 1997.

76. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell* 61: 203–212, 1990.

77. Voleti PB, Buckley MR, Soslowsky LJ. Tendon Healing: Repair and Regeneration. *Annu Rev Biomed Eng* 14: 47–71, 2012.

78. Wang JHC. Mechanobiology of tendon. *Journal of Biomechanics* 39: 1563–1582, 2006.

79. Wenstrup RJ, Florer JB, Brunskill EW, Bell SM, Chervoneva I, Birk DE. Type V Collagen Controls the Initiation of Collagen Fibril Assembly. *J Biol Chem* 279: 53331–53337, 2004.

80. Woo SL, Ritter MA, Amiel D, Sanders TM, Gomez MA, Kuei SC, Garfin SR, Akeson WH. The biomechanical and biochemical properties of swine tendons--long term effects of exercise on the digital extensors. *Connect Tissue Res* 7: 177–183, 1980.

81. Yoon JH, Halper J. Tendon proteoglycans: biochemistry and function. *J*

Musculoskelet Neuronal Interact 5: 22–34, 2005.

82. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 12: 21–35, 2010.

Chapter II

Changes in Macrophage Phenotype and Induction of Epithelial-to-Mesenchymal Transition Genes Following Acute Achilles Tenotomy and Repair

Abstract

Tendon injuries occur frequently in physically active individuals, but the clinical outcomes for these injuries can be poor. In many injured tissues the repair process is orchestrated by two types of cells, macrophages and fibroblasts. Macrophages, which have both pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes, can directly participate in tissue remodeling and direct the response of other cells through the secretion of cytokines and growth factors. In many organ systems, epithelial cells can transdifferentiate into fibroblasts, which can then regenerate damaged ECM. This process is triggered via activation of epithelial-to-mesenchymal transition (EMT) signaling programs. Most tendons are surrounded by sheets of epithelial cells, and these tissue layers could provide a source of fibroblasts to repair injured tendons. To gain greater insight into the biology of tendon repair, we performed a tenotomy and repair in Achilles tendons of adult rats and determined changes in macrophage phenotype and ECM- and EMT-related genes over a 4-week time course. The results from this study suggest that changes in macrophage phenotype and activation of EMT-

related programs likely contribute to the degradation and subsequent repair of injured tendon tissue.

Introduction

Tendon is a dynamic tissue that promotes efficient force transmission and drives locomotion by connecting muscle to bone. Injuries and diseases of tendons produce significant morbidity in patients, including debilitating pain and a decrease in functional capacity (48). While the etiology of tendon injury remains unclear, the accumulation of degenerative changes in the tendon extracellular matrix (ECM) over time predisposes the patient to spontaneous tendon rupture (48, 58). Repetitive microtrauma is thought to overwhelm the ability of tendon fibroblasts to repair the injured ECM network, and this likely leads to variegated cellularity, increased vascular elements and collagen fibril disorganization(9, 48). Indeed, degenerative tendinopathy is the most common histological finding at the site of tendon rupture, and reversal of this process can be challenging because tendons are particularly slow to heal (21). Accordingly, the management of tendon injuries is an exigent task for clinicians, and in many cases, patients remain symptomatic despite optimal therapy (58). In order to improve the treatment of tendon injuries, a greater understanding of the cellular and molecular factors that drive tendon healing is required.

In many injured tissues, the repair process proceeds through three overlapping stages, described as the inflammatory, proliferative and remodeling phases (17, 58). Although numerous cell types play important roles in tendon healing, the repair of injured tendon tissue is thought to be orchestrated by two types of cells, macrophages

and fibroblasts (48). Following the initial recruitment of neutrophils to the site of injury, an accumulation of macrophages is observed in the tendon ECM within the first 24 hours (33). Macrophages participate in the phagocytic removal of necrotic debris, but eventually shift their function to promote fibroblast proliferation and guide ECM remodeling through the release of chemotactic and growth factors (12, 17, 26, 58). Macrophages have two general phenotypes – classically activated pro-inflammatory macrophages (M1) that promote ECM breakdown, inflammation and apoptosis; and alternatively activated anti-inflammatory macrophages (M2) that coordinate ECM deposition and tissue repair (25, 49). Previous work in damaged skeletal muscle has demonstrated that suppression of either M1 or M2 macrophage function severely impairs tissue regeneration, indicating the important balance between inflammation and regeneration for proper tissue repair (31).

Fibroblasts are the predominant cell type in tendons and are responsible for the development, maintenance, repair and modification of the tendon ECM (24). In response to tissue injury, fibroblasts synthesize ECM proteins, including collagens, proteoglycans and glycoproteins (24). Fibroblasts also release matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes that selectively degrade various components of the ECM network, and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (9). The levels of MMPs and TIMPs fluctuate throughout the repair process, which culminates in the eventual replacement of the damaged tendon ECM with newly synthesized collagen and non-collagenous proteins (24, 48, 58). Previous work has demonstrated that the epitenon, a loose epithelial-like tissue layer that surrounds the tendon, may serve as a source of

fibroblasts contributing to tendon growth and repair (35, 53). In other organ systems, reactivation of one or more members of the Snail family of transcription factors promotes the transdifferentiation of epithelial cells to a mesenchymal cell lineage through a process known as epithelial-to-mesenchymal transition (EMT) (19, 23, 27). Once in the mesenchymal lineage, the cells adopt a fibroblast phenotype, and begin remodeling and synthesizing new ECM (19, 23, 27). While EMT plays an important part in the regeneration of other tissues, the role of the EMT process in tendon repair has not been previously described.

To gain greater understanding of the fundamental cellular and molecular biology of tendon repair, we performed a tenotomy and repair in Achilles tendons of adult rats and analyzed changes in macrophage phenotype and ECM- and EMT-related genes. Tissue was harvested at 3, 7, 14 and 28 days after injury and compared to uninjured controls. We hypothesized that in response to a full-thickness tear of the Achilles tendon there would be an early accumulation of M1 macrophages within the first week followed by gradual transition to the M2 phenotype, and that tendon repair would correlate with increased expression of EMT-related genes.

Methods

Animals and Surgical Procedure. The University of Michigan IACUC approved this study. Six-month-old male Sprague–Dawley rats were randomized to control, 3-, 7-, 14- or 28-day groups (N=6 rats per group). Tendon surgery was performed on both Achilles tendons. Rats were anesthetized with 2% isoflurane, and the skin overlying the surgical site was shaved and scrubbed with 4% chlorhexidine. A midline incision was

created in the skin and the paratenon was split to achieve visualization of the Achilles tendon. A full-thickness tenotomy was performed in the midsubstance of the tendon followed by immediate two-strand repair using the Bunnell technique with 5-0 Ethibond (Ethicon, Somerville, NJ) (Figure 2.1). The plantaris tendon was left intact. The paratenon was loosely re-approximated and the skin closed using 4-0 Vicryl (Ethicon) and GLUture (Abbott, Abbott Park, IL). A splash block of 0.5 ml of 0.5% bupivacaine, buprenorphine (0.05 mg/kg) and carprofen (5 mg/kg) were administered for analgesia during postoperative recovery. Ad libitum weight-bearing and cage activity were allowed. At harvest, rats were anesthetized with pentobarbital (50 mg/kg) and Achilles tendons were harvested for either gene expression analysis or immunohistochemistry (IHC). Achilles tendons were also collected from rats that did not undergo tenotomy and served as controls. After the removal of tendons, animals were euthanized by anesthetic overdose and induction of bilateral pneumothorax.

Gene Expression. The right Achilles tendon was homogenized in QIAzol (Qiagen, Valencia, CA). RNA was isolated using a miRNeasy Kit (Qiagen), treated with DNase I (Qiagen) and reverse transcribed into cDNA using oligo-dT_{15} and random hexamer primers with the RT^2 Kit (Qiagen). cDNA was amplified in a CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA) using a QuantiTect SYBR Green (Qiagen). The methods of Livak and Schmittgen (44) were used to normalize target gene expression to the stable housekeeping gene β2-microglobulin (B2M). Transcript information and the specific function of each gene are provided in Table 2.1.

Immunohistochemistry. Left Achilles tendons were isolated, snap frozen in TFM (Triangle Biosciences, Durham, NC), and stored at -80°C. Tendons were sectioned

through the callus at a thickness of 10 μm in a cryostat. Slides were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 and blocked with 5% goat serum. Slides were incubated with primary antibodies against rabbit anti-CCR7 (NB110-55680, Novus Biologicals, Littleton, CO) and mouse anti-CD163 (MCA342B, AbD Serotec, Raleigh, NC) to label M1 and M2 macrophages, respectively (2, 7). Alexa Fluor 555 (AF555, Life Technologies, Eugene, OR) secondary antibodies or streptavidin-AF647 were used to detect primary antibodies. DAPI was used to identify nuclei and ECM was labeled with WGA-lectin conjugated to AF488 (Life Technologies). Slides were mounted and imaged using a Zeiss Axiovert 200M outfitted with the ApoTome system (Carl Zeiss, Thornwood, NY).

Statistical Analyses. Results are presented as mean±SE. Prism 6.0 software (GraphPad Software, La Jolla, CA) was used to conduct analyses. A one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting was performed to determine significance between groups.

Results

Control tendons demonstrated the presence of M2 and occasional M1 macrophages in the endotenon layers, but no macrophages in the tendon fibers (Figure 2.2A). Three days following surgical tear and repair, M1 macrophages accumulated in regions of newly formed tendon tissue and remained present throughout the study period (Figure 2.2B-E). M2 macrophages slowly accumulated at sites of organizing tendon ECM and became the predominant macrophage phenotype by 28 days (Figure 2.2E). In most cases, after injury macrophages were localized in areas of tissue

resorption as indicated by the lack of WGA staining. The change in M1 and M2 signal present in IHC was accompanied by quantitative changes in gene expression. For genes that regulate macrophage and neutrophil function (Figure 2.3), the neutrophil marker Ly6c was elevated by 3 days, but returned to levels similar to controls by 28 days. The pan-macrophage marker F4/80 was also elevated by 3 days. However, after an initial decline at 7 days, it remained elevated compared to controls thereafter. CCL2, which plays an important role in macrophage recruitment, was elevated by 3 days and then steadily declined over the next few weeks. Markers of M1 macrophages such as CD68, CCR7 and CD11b were dramatically elevated following surgical tear and repair. CD68 and CD11b declined between 3 and 7 days. CD168, a marker for M2 macrophages, remained similar to controls until day 28 at which time it became significantly elevated. Pro-inflammatory interleukins IL1b and IL6 were up-regulated by 3 days, but IL6 demonstrated a gradual decline in expression between each time point. Upregulation of the anti-inflammatory interleukin IL10 did not occur until 28 days.

For fibroblast proliferation and cell cycle control factors (Figure 2.4), the type II transmembrane protein tenomodulin (Tnmd) promotes tendon fibroblast proliferation and was significantly downregulated by 3 days. It was then elevated compared to controls by 7 days with peak expression at 14 days. Scleraxis (Scx), a bHLH transcription factor involved in tendon development, was elevated by 7 days with a slight decline in expression at 28 days. Alpha smooth muscle actin (SMA) is an indicator of fibroblast contractility and was elevated at 7 days, but quickly returned to levels similar to controls by 14 days. No changes in the pan-fibroblast marker FSP1 were noted at any time point. Moreover, no differences in Mohawk (Mkx) expression, a

marker of tendon maturation, were observed. HIF1a serves as a gauge of tissue hypoxia and was elevated by 3 days with further increases in expression at 7 and 14 days. Egr1 and Egr2, transcriptional regulatory proteins that are induced by various mitogenic cues, were elevated by 7 days. While Egr1 returned to levels similar to controls by 14 days, Egr2 remained elevated for the remainder of the study period. The cellular differentiation marker Aatk remained similar to controls until day 28 at which time its expression was upregulated.

For EMT-related genes (Figure 2.5), the transcription factors Snail1 (Snai1) and Slug were elevated by 3 days and then declined by either 14 or 28 days, respectively. Goosecoid (Gsc), a homeobox protein that induces EMT, was elevated at 7 and 14 days compared to controls. Twist1, a bHLH transcription factor that also induces EMT, was elevated by 7 days and returned to baseline levels by 28 days. No differences in expression of the angiogenic factor VEGF or the transcription factor Foxc2, which is induced by EMT events, were noted at any time point. The endothelial cell marker CD31 was elevated at 7 days and the cell adhesion molecule E-cadherin (Cdh1) was significantly upregulated at 28 days. Ddr2, an EMT-responsive receptor tyrosine kinase, was downregulated by 3 days, but returned to levels similar to controls by 7 days.

For ECM gene expression (Figure 2.6), the small leucine-rich proteoglycan (SLRP), biglycan (Bgn), and the cartilage-specific proteoglycan aggrecan (Acan) were significantly elevated by 3 and 7 days, respectively. Both remained elevated with a slight decrease between 14 and 28 days. In contrast, the SLRPs decorin (Dcn), and fibromodulin (Fmod) were downregulated by 3 days, and only Fmod returned to levels similar to controls by 7 days. The intermediate filament vimentin (Vim) was briefly

upregulated at 7 days, while the large proteoglycan versican (Vcan) was significantly expressed by 3 days and then steadily declined. Collagen type I (Col1a1) and type III (Col3a1) were both upregulated by 3 days with peak expression at 7 days. Members of the MMP family displayed similar levels of expression with upregulation of MMP2, MMP3, MMP8 and MMP14 by 3 days, and MMP9 by 7 days. MMP2, MMP8 and MMP14 were maximally expressed at 14 days and declined thereafter, while MMP9 steadily increased over the next few weeks. TIMP1 was elevated by 3 days and then declined by 14 days, whereas TIMP2 was elevated by 7 days with peak expression at 14 days.

Discussion

While tendon injuries are common and can significantly detract from the quality of life, compared with other musculoskeletal tissues little is known about the biology of tendon repair. This was the first study, to our knowledge, that identified changes in canonical EMT-related genes over the course of tendon injury and repair. Further, we report changes in different populations of macrophages, and in fibroblast proliferation and ECM synthesis genes that play important structural and mechanical roles in tendon. There appears to be overlapping activation of various gene families within the first four weeks of tendon repair, and EMT-related factors may contribute to the repair process in injured tendon tissue.

In response to structural damage, chemotactic cytokines that promote the delivery of different cell populations to the repair site are released from the tendon ECM (9, 24). Phagocytic neutrophils and M1 macrophages are the first to arrive within 24

hours after injury, followed by a reparative shift in function that correlates with increases in M2 macrophages (17, 48, 58). Using ED1 (CD68) and ED2 (CD163) to identify pro- and anti-inflammatory macrophages, respectively, Marsolais et al. (33) found that following the injection of collagenase into tendons, there was a rapid accumulation of M1 macrophages that returned to baseline levels two weeks after injury. No significant change in M2 macrophages was detected over the course of the study. In a study that explored early mechanical loading after collagenase injection into tendons, Godbout et al. (14) reported an increase in M1 macrophages that was similar to Marsolais et al. (33), but also that exercise increased M2 macrophages three days after injury. The results of the current study are consistent with previous work as evidenced by upregulation of M1 markers CD68, CCR7 and CD11b as well as interleukins IL1b and IL6 within 72 hours. These pro-inflammatory factors remained consistently elevated at each time point, while the M2 marker CD168 and interleukin IL10 were not induced until 28 days. Similar observations in the early M1 response were found in injured skeletal muscle using a model of reloading after hindlimb suspension (51, 55). Following resumption of weight-bearing, M1 macrophages precipitously accumulate in the reloaded muscle within 48 hours, but expression was more transient with the M1 signal returning to levels similar to controls within one week post-injury. Although M2 macrophages also appeared at much earlier time points, the sequential transition between the M1 and M2 phenotypes supports the dual functionality of this cell type in the degradation and repair of injured tendons.

Following connective tissue injury, there is a pronounced early increase in the number of macrophages and fibroblasts (48). While tendon fibroblasts are thought to

come from either a circulating pool of bone marrow-derived progenitor cells or from resident populations of tendon stem/progenitor cells (4), a growing body of evidence in other tissues suggests that resident cells in the host epithelium become activated and undergo an EMT transcriptional reprogramming to a mesenchymal fibroblast phenotype (19, 23). As cells undergo EMT, they experience a loss of epithelial-associated junctional complexes, begin to express mesenchymal cell markers and acquire tissue-invasive properties that allow them to transmigrate their basement membrane and move into different environments. The epitenon is an epithelial structure subtended by a basement membrane (53), and prior work in in vitro and in vivo models of lacerated flexor tendons has demonstrated that fibroblasts migrate from this outer layer into the site of tendon injury (13, 22, 32, 53), as well as in response to treadmill training (35). In the present study, we found that following acute Achilles tenotomy and repair there was a significant upregulation of canonical EMT-related genes including Snail1, Slug, Goosecoid and Twist1. These transcription factors repress cell adhesion proteins and induce metalloproteinases that degrade the ECM and promote tissue invasion (37). Accompanying these changes were increased expression of mesenchymal tendon fibroblast markers scleraxis and tenomodulin, as well as ECM-remodeling enzymes MMP2, MMP3, MMP8, MMP9 and MMP14, and their endogenous inhibitors TIMP1 and TIMP2. Consistent with these results, other models of Achilles tendon injury have demonstrated massive increases in the expression of tendon-specific markers (11, 20). Moreover, Scott and colleagues (46) found that scleraxis, tenomodulin and type I collagen were significantly expressed in an injured mouse patellar tendon 4 weeks after a central defect was surgically created. Scleraxis and type I collagen expression

eventually returned to baseline levels, but tenomodulin remained elevated even at 12 weeks post-injury. Induction of MMP expression has also been shown to correlate with collagen turnover and ECM remodeling during tendon repair (9, 40). Further, proteoglycans are important regulators of collagen fibril assembly and variations in their content can greatly alter tendon structure and function (10). Following surgical tear and repair of the Achilles tendon, we observed an upregulation of type I and III collagens, aggrecan, biglycan and versican, and a downregulation of decorin and fibromodulin. The reduction in decorin expression is supported by previous studies that demonstrated decreased rates of collagen fibrillogenesis in vitro in the presence of decorin (8, 57). In addition to abnormal fibril structure and impaired mechanical function, decorin-deficient tendons display an increase in biglycan expression (60). This may compensate for the loss of decorin as biglycan is expressed during early tendon development, but its expression normally declines as the tendon matures (1). Similar to the altered tendon phenotype described in decorin-null mice, a deficiency of fibromodulin also produces tendons with thin, irregular collagen fibrils (52). Aggrecan is a proteoglycan, that is expressed during later stages of chondrogenesis, and is also expressed in regenerating tendons following tenotomy (29). Increased expression of aggrecan is correlated with the induction of heterotopic ossification in injured tendons (29), and although we did not directly measure other markers of osteogenesis, the increase in aggrecan at later time points suggests some heterotopic ossification was also occurring in our model. Overall, findings from this study in conjunction with previous work suggest that the assembly of the collagen network during tendon repair may be mediated by the temporal-specific expression of proteoglycans.

This study provided important insights into changes in macrophage phenotype and EMT-related genes following acute Achilles tenotomy and repair, but there are several limitations. We did not immobilize the hindlimbs postoperatively, which is standard practice in patients undergoing Achilles tendon repairs. However, the plantaris tendon was left intact, and as the plantaris tendon is substantially larger in rats than humans, this likely provides stress shielding for the injured Achilles tendon. Immediate mobilization at the repair site may have impacted the amount of inflammation present, but should not have altered the sequence of inflammatory cell accumulation within the injured tissue. We also focused only on the Achilles tendon and the observed differences may not be reflected in trunk or other limb tendons. While there are 26 known MMP genes (9), we evaluated only a subset of MMPs that were selected from the four general classes of MMPs. Our animal model is most representative of acute tendon transections, and may not recapitulate longstanding degenerative changes in the tendon ECM. Finally, in addition to driving transdifferentiation and migration through the EMT program, Snail1, Slug, Goosecoid and Twist1 have other EMT-independent functions including cell lineage specification, cell cycle regulation, apoptosis, and both ECM synthesis and remodeling (3, 41, 47, 56). While these results are encouraging, whether EMT is truly occurring in tendon, or fibroblasts emerging from the epitenon are simply proliferating populations of resident cells, remains to be determined. Further studies are needed to decipher the specific roles of these genes during tendon repair. This study evaluated changes in the expression of EMT-related genes during the repair of injured tendon tissue, but changes in the levels of specific proteins were not investigated. However, previous studies in other tissues have demonstrated that

changes in the expression of specific EMT-related genes correlate with changes in the abundance of the translated proteins (34, 42).

Injuries to and diseases of tendons are a substantial economic and health burden among patients of all ages. Nonoperative interventions focus on symptomatic relief, and even with surgery patients may have poor outcomes and continue to experience pain and reduced function (48). A more direct approach to the management of tendon injuries is needed, and the current lack of targeted therapies is due in part to our limited mechanistic insight into basic physiological processes that control tendon repair. Findings from this study and from Marsolais et al. (33) demonstrate that macrophages change phenotype in response to tendon damage and this likely contributes to the degradation and subsequent repair of injured tendon ECM. Further investigation of the specific functions of macrophage sub-populations in tendon is therefore warranted. Lastly, given the critical role EMT plays in the postnatal growth of many tissues in the body and in pathologic conditions such as tissue fibrosis, the expression of EMT-related genes during tendon repair may prove to be a worthwhile area of scientific exploration.

Acknowledgements

The authors would like to acknowledge helpful discussions with Dr. Stephen J Weiss. This work was supported by NIH grants R01-AR063649, T32-GM008616 and T32-GM008322. This chapter represents previously published work: Sugg KB, Lubardic J, Gumucio JP, Mendias CL. Changes in macrophage phenotype and induction of epithelial-to-mesenchymal transition genes following acute Achilles tenotomy and repair. *J Orthop Res*. 2014;32(7):944-51.

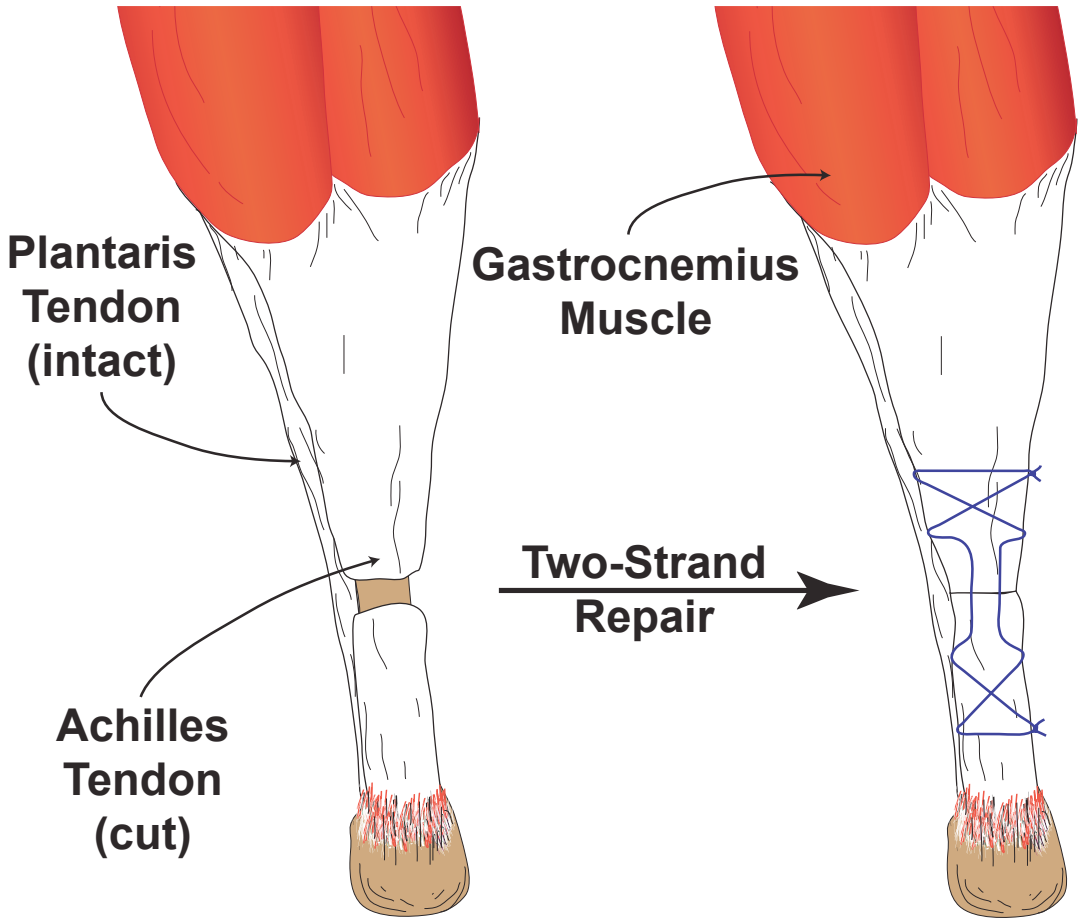

Figure 2.1. Illustration of tendon repair. A full-thickness tenotomy was performed in the midsubstance of the Achilles tendon and the plantaris tendon was left intact. The defect was then repaired immediately using a two-strand core suture (Bunnell technique).

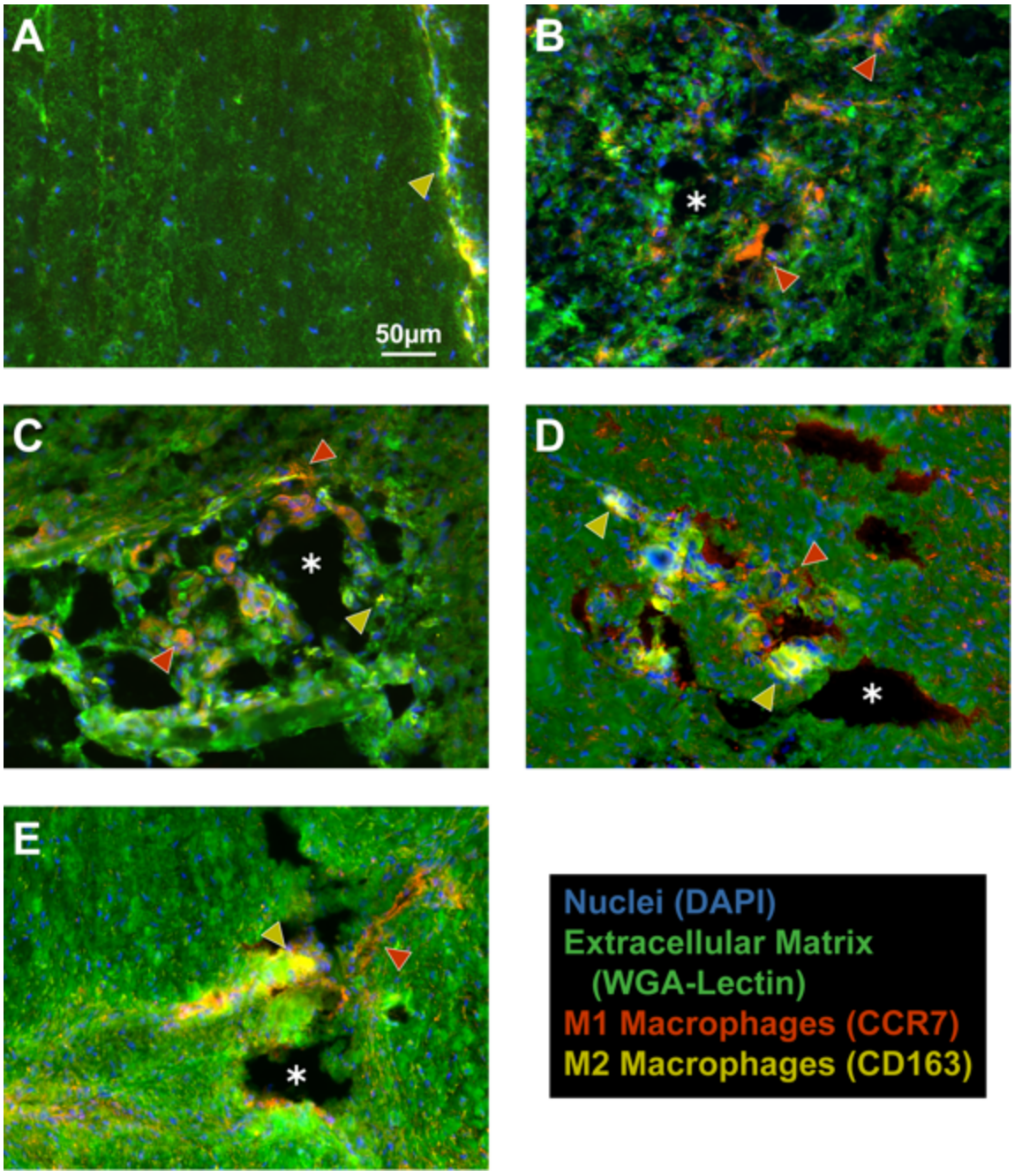

Figure 2.2. Macrophages accumulate sequentially in tendon following surgical tear and repair. M2 macrophages are found in the endotenon of (A) nonoperated controls, but not in the tendon fibers. There is a dramatic accumulation of M1 macrophages in regions of ECM resorption that remained consistent in the (B) 3-day, (C) 7-day and (D) 14-day groups. M2 macrophages become the predominant phenotype by (E) 28 days. M1 macrophages (CCR7), red (and indicated by red arrowheads); M2 macrophages (CD163), yellow (and indicated by yellow arrowheads); ECM (WGA-lectin), green; nuclei (DAPI), blue. Asterisks indicate areas of tissue resorption. All panels are shown at the same level of magnification as in panel A.

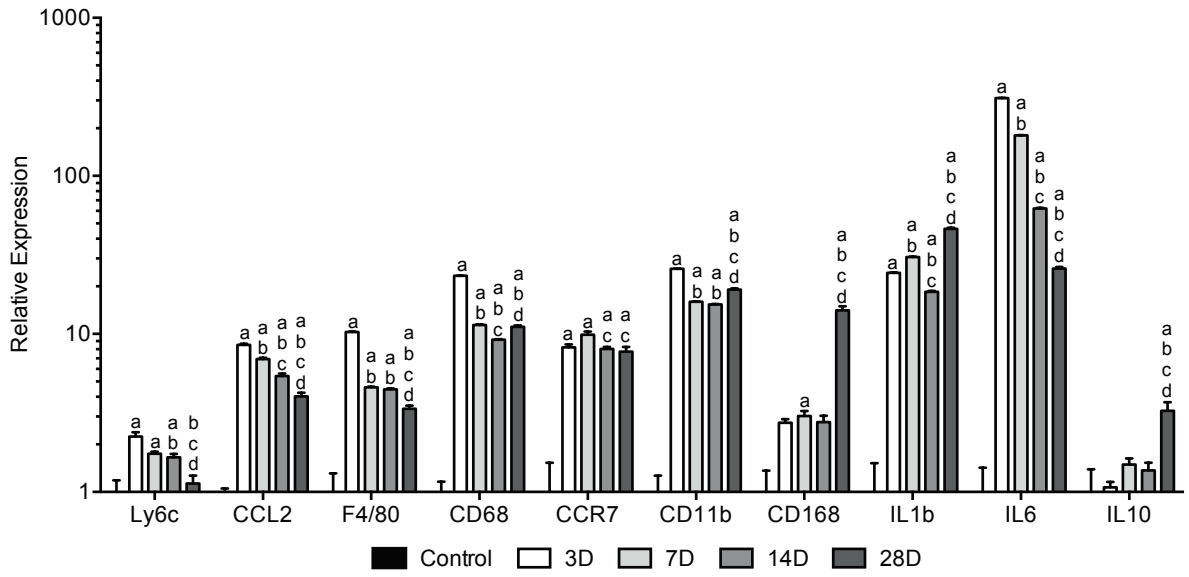

Figure 2.3. Changes in gene expression of macrophage and neutrophil markers in the Achilles tendon following surgical tear and repair. Target genes were normalized to B2M expression, and further normalized to the control group. Differences were tested using a one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting. (a) Different from control, (b) different from 3D, (c) different from 7D and (d) different from 14D. N=6 tendons per group.

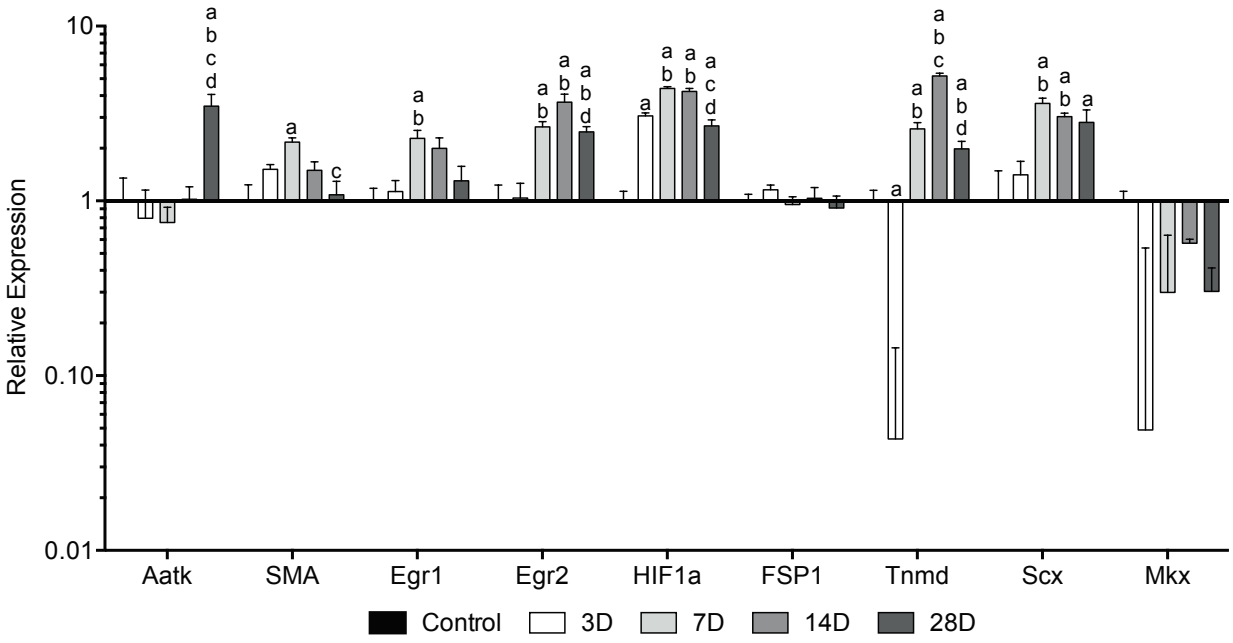

Figure 2.4. Changes in gene expression of fibroblast proliferation and cell cycle control factors in the Achilles tendon following surgical tear and repair. Target genes were normalized to B2M expression, and further normalized to the control group. Differences were tested using a one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting. (a) Different from control, (b) different from 3D, (c) different from 7D and (d) different from 14D. N=6 tendons per group.

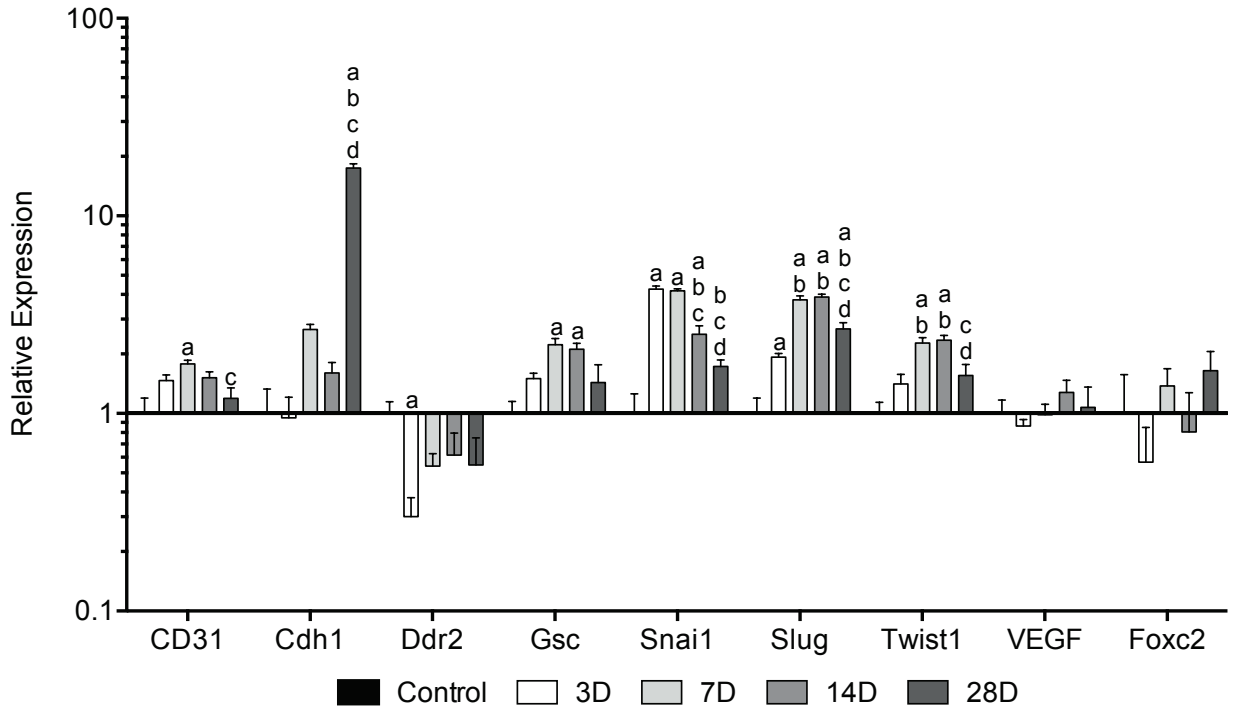

Figure 2.5. Changes in gene expression of EMT-related genes in the Achilles tendon following surgical tear and repair. Target genes were normalized to B2M expression, and further normalized to the control group. Differences were tested using a one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting. (a) Different from control, (b) different from 3D, (c) different from 7D and (d) different from 14D. N=6 tendons per group.

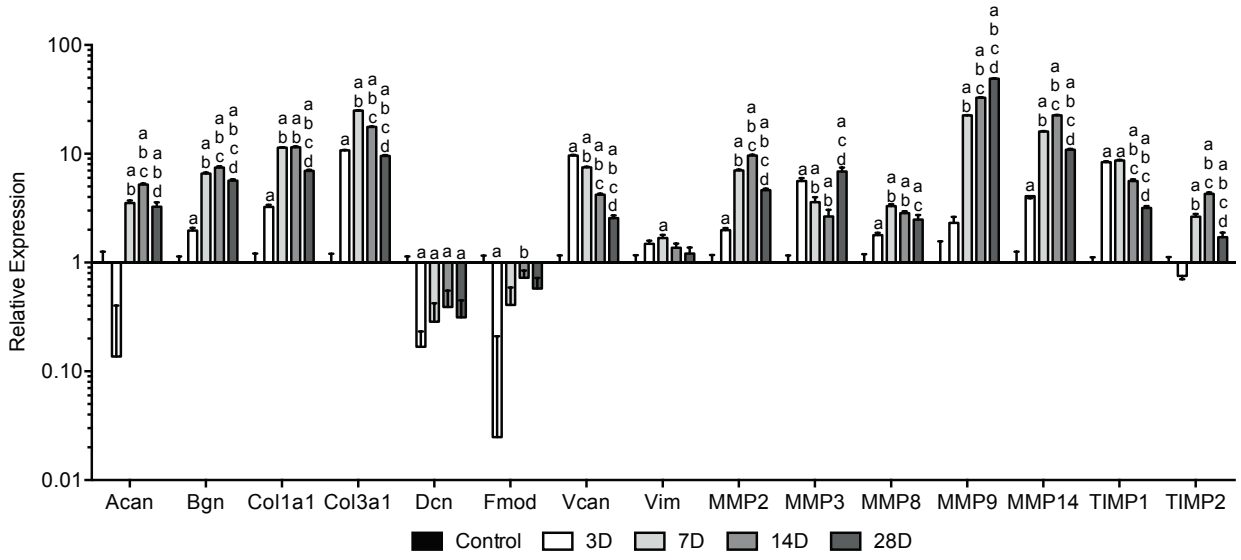

Figure 2.6. Changes in gene expression of ECM components and MMPs in the Achilles tendon following surgical tear and repair. Target genes were normalized to B2M expression, and further normalized to the control group. Differences were tested using a one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting. (a) Different from control, (b) different from 3D, (c) different from 7D and (d) different from 14D. N=6 tendons per group.

mRNA	Description / Purpose	RefSeq No.
Macrophage / Neutrophil		
Ly6c	Ly6-C antigen / Neutrophil marker (36)	NM_020103
CCL2	Chemokine (C-C motif) ligand 2 / Macrophage recruitment marker (38)	NM_031530
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1 / Pan-macrophage marker (16)	NM_001007557
CD68	Cd68 molecule / M1 macrophage marker (16)	NM_001031638
CCR7	Chemokine (C-C motif) receptor 7 / M1 macrophage marker (16)	NM_199489
CD11b	Integrin alpha M / M1 macrophage marker (16)	AF268593
CD168	Hyaluronan-mediated motility receptor (RHAMM) / M2 macrophage marker (16)	NM_012964
IL1b	Interleukin 1 beta / Pro-inflammatory marker (16)	NM_031512
IL6	Interleukin 6 / Pro-inflammatory marker (16)	NM_012589
IL10	Interleukin 10 / Anti-inflammatory marker (16)	NM_012854
Fibroblast Proliferation / Cell Cycle Control		
Aatk	Apoptosis-associated tyrosine kinase / Cellular differentiation marker (39)	NM_001168703
SMA	Alpha-smooth muscle actin / Marker of fibroblast contractility (61)	NM_031004
Egr1	Early growth response 1 / Transcriptional regulatory protein (15)	AY551092
Egr2	Early growth response 2 / Transcriptional regulatory protein (28)	NM_053633
HIF1a	Hypoxia-inducible factor 1, alpha subunit / Tissue hypoxia marker (61)	NM_024359
FSP1	Fibroblast specific protein 1, S100 calcium-binding protein A4 / Pan-fibroblast marker (54)	NM_012618
Tnmd	Tenomodulin / Marker of tendon fibroblast proliferation (35)	NM_022290
Scx	Scleraxis / Marker of tendon development (35)	NM_001130508
Mkx	Mohawk homeobox / Marker of tendon maturation (18)	XM_001063892
EMT-Related Genes		
CD31	Platelet endothelial cell adhesion molecule 1 / Endothelial cell marker (61)	NM_031591
Cdh1	Cadherin 1, E-cadherin / Epithelial cell adhesion molecule (37)	NM_031334
Ddr2	Discoidin domain receptor tyrosine kinase 2 / EMT-responsive receptor tyrosine kinase (59)	NM_031764
Gsc	Goosecoid homeobox / Homeobox protein (37)	NM_001191873
Snai1	Snail homolog 1 (Drosophila) / Zinc-finger transcription factor (37)	NM_053805
Slug	Snail homolog 2 (Drosophila) / Zinc-finger transcription factor (37)	NM_013035
Twist1	Twist homolog 1 (Drosophila) / bHLH transcription factor (37)	NM_053530
VEGF	Vascular endothelial growth factor A / Angiogenic factor (61)	NM_031836
Foxc2	Forkhead box C2 / Forkhead box transcription factor (30)	NM_001101680
ECM Components / MMPs		
Acan	Aggrecan / Cartilage-specific proteoglycan (29)	NM_022190
Bgn	Biglycan / Small leucine-rich proteoglycan (SLRP) (4)	NM_017087
Col1a1	Type I collagen / Mature collagen marker (9)	NM_053304
Col3a1	Type III collagen / Immature collagen marker (9)	NM_032085
Dcn	Decorin / Small leucine-rich proteoglycan (SLRP) (10)	NM_024129
Fmod	Fibromodulin / Small leucine-rich proteoglycan (SLRP) (4)	NM_080698
Vcan	Versican / Large proteoglycan (45)	NM_001170558
Vim	Vimentin / Intermediate filament (5)	NM_031140
MMP2	Matrix metalloproteinase 2 / Gelatinase A (9)	NM_031054
MMP3	Matrix metalloproteinase 3 / Stromelysin-1 (50)	NM_133523
MMP8	Matrix metalloproteinase 8 / Collagenase-2 (50)	NM_022221
MMP9	Matrix metalloproteinase 9 / Gelatinase B (9)	NM_031055
MMP14	Matrix metalloproteinase 14 / Membrane-tethered type-1 matrix metalloproteinase (9)	NM_031056
TIMP1	Tissue inhibitor of metalloproteinase 1 / Inhibitor of secreted MMPs (43)	NM_053819
TIMP2	Tissue inhibitor of metalloproteinase 2 / Inhibitor of secreted and membrane-anchored MMPs (43)	NM_021989
Housekeeping		
B2M	Beta-2 microglobulin / Component of class I MHC molecules (6)	NM_012512

Table 2.1. mRNA transcripts evaluated by quantitative PCR.

References

1. Ansorge HL, Adams S, Birk DE, Soslowsky LJ. Mechanical, Compositional, and Structural Properties of the Post-natal Mouse Achilles Tendon. *Ann Biomed Eng* 39: 1904–1913, 2011.

2. Badylak SF, Valentin JE, Ravindra AK, McCabe GP, Stewart-Akers AM. Macrophage Phenotype as a Determinant of Biologic Scaffold Remodeling. *Tissue Engineering Part A* 14: 1835–1842, 2008.

3. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132: 3151–3161, 2005.

4. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, Li L, Leet AI, Seo B-M, Zhang L, Shi S, Young MF. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 13: 1219–1227, 2007.

5. Bornemann A, Schmalbruch H. Desmin and vimentin in regenerating muscles. *Muscle Nerve* 15: 14–20, 1992.

6. Calve S, Isaac J, Gumucio JP, Mendias CL. Hyaluronic acid, HAS1, and HAS2 are significantly upregulated during muscle hypertrophy. *AJP: Cell Physiology* 303: C577–C588, 2012.

7. Chin L, Calabro A, Rodriguez ER, Tan CD, Walker E, Derwin KA. Characterization of and host response to tyramine substituted-hyaluronan enriched fascia extracellular matrix. *J Mater Sci: Mater Med* 22: 1465–1477, 2011.

8. Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *The Journal of Cell Biology* 136: 729–743, 1997.

9. Davis ME, Gumucio JP, Sugg KB, Bedi A, Mendias CL. MMP inhibition as a potential method to augment the healing of skeletal muscle and tendon extracellular matrix. *J Appl Physiol* 115: 884–891, 2013.

10. Dunkman AA, Buckley MR, Mienaltowski MJ, Adams SM, Thomas SJ, Satchell L, Kumar A, Pathmanathan L, Beason DP, Iozzo RV, Birk DE, Soslowsky LJ. Matrix Biology. *Matrix Biol* 32: 3–13, 2013.

11. Eliasson P, Andersson T, Aspenberg P. Rat Achilles tendon healing: mechanical loading and gene expression. *Journal of Applied Physiology* 107: 399–407, 2009.

12. Fukasawa M, Bryant SM, Nakamura RM, diZerega GS. Modulation of fibroblast proliferation by postsurgical macrophages. *J Surg Res* 43: 513–520, 1987.

13. Gelberman RHR, Steinberg DD, Amiel DD, Akeson WW. Fibroblast chemotaxis after tendon repair. *J Hand Surg Am* 16: 686–693, 1991.

14. Godbout C, Ang O, Frenette J. Early voluntary exercise does not promote healing in a rat model of Achilles tendon injury. *Journal of Applied Physiology* 101: 1720–1726, 2005.

15. Guerquin M-J, Charvet B, Nourissat G, Havis E, Ronsin O, Bonnin M-A, Ruggiu M, Olivera-Martinez I, Robert N, Lu Y, Kadler KE, Baumberger T, Doursounian L, Berenbaum F, Duprez D. Transcription factor EGR1 directs tendon differentiation and promotes tendon repair. *J Clin Invest* 123: 3564–3576, 2013.

16. Gumucio JP, Korn MA, Saripalli AL, Flood MD, Phan AC, Roche SM, Lynch EB, Claflin DR, Bedi A, Mendias CL. Aging-associated exacerbation in fatty degeneration and infiltration after rotator cuff tear. *Journal of Shoulder and Elbow Surgery* 23: 99–108, 2014.

17. Hope M, Saxby TS. Tendon Healing. *Foot and Ankle Clinics* 12: 553–567, 2007.

18. Ito Y, Toriuchi N, Yoshitaka T, Ueno-Kudoh H, Sato T, Yokoyama S, Nishida K, Akimoto T, Takahashi M, Miyaki S, Asahara H. The Mohawk homeobox gene is a critical regulator of tendon differentiation. *Proc Natl Acad Sci USA* 107: 10538–10542, 2010.

19. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 110: 341–350, 2002.

20. James R, Kesturu G, Balian G, Chhabra AB. Tendon: Biology, Biomechanics, Repair, Growth Factors, and Evolving Treatment Options. *J Hand Surg Am* 33: 102–112, 2008.

21. Järvinen M, Jozsa L, Kannus P, Järvinen TLN, Kvist M, Leadbetter W. Histopathological findings in chronic tendon disorders. *Scandinavian Journal of Medicine & Science in Sports* 7: 86–95, 1997.

22. Jones ME, Mudera V, Brown RA, Cambrey AD, Grobbelaar AO, McGrouther DA. The early surface cell response to flexor tendon injury. *J Hand Surg Am* 28: 221–230, 2003.

23. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 119: 1420–1428, 2009.

24. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiological Reviews* 84: 649–698, 2004.

25. Laskin DL. Macrophages and Inflammatory Mediators in Chemical Toxicity: A Battle of Forces. *Chem Res Toxicol* 22: 1376–1385, 2009.

26. Leadbetter WB. Cell-matrix response in tendon injury. *Clinics in Sports Medicine*.

27. Lee JM. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *The Journal of Cell Biology* 172: 973–981, 2006.

28. Léjard V, Blais F, Guerquin M-J, Bonnet A, Bonnin M-A, Havis E, Malbouyres M, Bidaud CB, Maro G, Gilardi-Hebenstreit P, Rossert J, Ruggiero F, Duprez D. EGR1 and EGR2 Involvement in Vertebrate Tendon Differentiation. *J Biol Chem* 286: 5855–5867, 2011.

29. Lin L, Shen Q, Xue T, Yu C. Heterotopic ossification induced by Achilles tenotomy via endochondral bone formation: Expression of bone and cartilage related genes. *Bone* 46: 425–431, 2010.

30. Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, Kutok JL, Hartwell K, Richardson AL, Weinberg RA. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci USA* 104: 10069–10074, 2007.

31. Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, Muñoz-Cánoves P. Aberrant repair and fibrosis development in skeletal muscle. *Skeletal Muscle* 1: 21, 2011.

32. Manske PRP, Lesker PAP. Histologic evidence of intrinsic flexor tendon repair in various experimental animals. An in vitro study. *Clin Orthop Relat Res* : 297–304, 1984.

33. Marsolais D, Cŏté CH, Frenette J. Neutrophils and macrophages accumulate sequentially following Achilles tendon injury. *J Orthop Res* 19: 1203–1209, 2001.

34. Medici D, Hay ED, Olsen BR. Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol Biol Cell* 19: 4875–4887, 2008.

35. Mendias CL, Gumucio JP, Bakhurin KI, Lynch EB, Brooks SV. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. *J Orthop Res* 30: 606–612, 2011.

36. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nature Reviews Immunology* 11: 723–737, 2011.

37. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 7: 415–428, 2007.

38. Qian B-Z, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, Pollard JW. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* 475: 222–225, 2011.

39. Raghunath M, Patti R, Bannerman P, Lee CM, Baker S, Sutton LN, Phillips PC, Damodar Reddy C. A novel kinase, AATYK induces and promotes neuronal differentiation in a human neuroblastoma (SH-SY5Y) cell line. *Brain Res Mol Brain Res* 77: 151–162, 2000.

40. Riley G. The pathogenesis of tendinopathy. A molecular perspective. *Rheumatology (Oxford)* 43: 131–142, 2004.

41. Rowe RG, Li XY, Hu Y, Saunders TL, Virtanen I, de Herreros AG, Becker KF, Ingvarsen S, Engelholm LH, Bommer GT, Fearon ER, Weiss SJ. Mesenchymal cells reactivate Snail1 expression to drive three-dimensional invasion programs. *The Journal of Cell Biology* 184: 399–408, 2009.

42. Rowe RG, Lin Y, Shimizu-Hirota R, Hanada S, Neilson EG, Greenson JK, Weiss SJ. Hepatocyte-Derived Snail1 Propagates Liver Fibrosis Progression. *Mol Cell Biol* 31: 2392–2403, 2011.

43. Sabeh F, Li X-Y, Saunders TL, Rowe RG, Weiss SJ. Secreted versus membrane-anchored collagenases: relative roles in fibroblast-dependent collagenolysis and invasion. *J Biol Chem* 284: 23001–23011, 2009.

44. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3: 1101–1108, 2008.

45. Scott A, Lian Ø, Roberts CR, Cook JL, Handley CJ, Bahr R, Samiric T, Ilic MZ, Parkinson J, Hart DA, Duronio V, Khan KM. Increased versican content is associated with tendinosis pathology in the patellar tendon of athletes with jumper's knee. *Scandinavian Journal of Medicine & Science in Sports* 18: 427–435, 2007.

46. Scott A, Sampaio A, Abraham T, Duronio C, Underhill TM. Scleraxis expression is coordinately regulated in a murine model of patellar tendon injury. *J Orthop Res* 29: 289–296, 2010.

47. Sekiya SS, Suzuki AA. Glycogen synthase kinase 3 β-dependent Snail degradation directs hepatocyte proliferation in normal liver regeneration. *Proc Natl Acad Sci USA* 108: 11175–11180, 2011.

48. Sharma PP, Maffulli NN. Tendon injury and tendinopathy: healing and repair. *J Bone Joint Surg Am* 87: 187–202, 2005.

49. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* 122: 787–795, 2012.

50. Snoek-van Beurden P, den Hoff Von JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques* 38: 73–83, 2005.

51. St Pierre BA, Tidball JG. Macrophage activation and muscle remodeling at myotendinous junctions after modifications in muscle loading. *Am J Pathol* 145: 1463–1471, 1994.

52. Svensson L, Aszódi A, Reinholt FP, Fassler R, Heinegård D, Oldberg A. Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon. *J Biol Chem* 274: 9636–9647, 1999.

53. Taylor SH, Al-Youha S, Van Agtmael T, Lu Y, Wong J, McGrouther DA, Kadler KE. Tendon Is Covered by a Basement Membrane Epithelium That Is Required for Cell Retention and the Prevention of Adhesion Formation. *PLoS ONE* 6: e16337, 2011.

54. Teng Y, Kanasaki K, Bardeesy N, Sugimoto H, Kalluri R. Deletion of Smad4 in fibroblasts leads to defective chondrocyte maturation and cartilage production in a TGFÎ² type II receptor independent manner. *Biochemical and Biophysical Research Communications* 407: 633–639, 2011.

55. Tidball JG. Mechanisms of muscle injury, repair, and regeneration. *Compr Physiol* 1: 2029–2062, 2011.

56. Vega S. Snail blocks the cell cycle and confers resistance to cell death. *Genes & Development* 18: 1131–1143, 2004.

57. Vogel KG, Paulsson M, Heinegård D. Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. *Biochem J* 223: 587–597, 1984.

58. Voleti PB, Buckley MR, Soslowsky LJ. Tendon Healing: Repair and Regeneration. *Annu Rev Biomed Eng* 14: 47–71, 2012.

59. Walsh LA, Nawshad A, Medici D. Discoidin domain receptor 2 is a critical regulator of epithelial–mesenchymal transition. *Matrix Biology* 30: 243–247, 2011.

60. Zhang G, Ezura Y, Chervoneva I, Robinson PS, Beason DP, Carine ET, Soslowsky LJ, Iozzo RV, Birk DE. Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development. *J Cell Biochem* 98: 1436–1449, 2006.

61. Zordan P, Rigamonti E, Freudenberg K, Conti V, Azzoni E, Rovere-Querini P, Brunelli S. Macrophages commit postnatal endothelium-derived progenitors to angiogenesis and restrict endothelial to mesenchymal transition during muscle regeneration. *Cell Death and Disease* 5: e1031–14, 2014.

Chapter IIII

Postnatal Tendon Growth and Remodeling Requires Platelet-Derived Growth Factor Receptor Signaling

Abstract

Platelet-derived growth factor receptor (PDGFR) signaling plays an important role in the specification and fundamental biological activities of many cells that compose musculoskeletal tissues, although little is known about PDGFR signaling during tendon growth and remodeling *in vivo*. Using the hindlimb synergist ablation model of tendon growth, our main objective was to determine the role of PDGFR signaling in the adaptation of tendons subjected to a mechanical growth stimulus, as well as to investigate the biological mechanisms behind this response. We demonstrate that both PDGFRs, PDGFRα and PDGFRβ, are expressed in tendon fibroblasts, and that the inhibition of PDGFR signaling suppresses the normal growth of tendon tissue after mechanical overload due to defects in fibroblast proliferation and migration. We also identify membrane type-1 matrix metalloproteinase (MT1-MMP) as an essential proteinase for fibroblast migration through tendon extracellular matrix. Furthermore, we show that MT1-MMP translation is regulated by PI3K/Akt, while ERK1/2 controls post-translational trafficking of MT1-MMP within tendon fibroblasts. Taken together, these

findings demonstrate that PDGFR signaling is necessary for postnatal tendon growth and remodeling, and that MT1-MMP is a critical mediator of tendon fibroblast migration and a potential target for the treatment of tendon injuries and diseases.

Introduction

Tendon is an integral component of the musculoskeletal system. Anatomically situated between skeletal muscle and bone, tendon transmits and stores force, allowing for efficient locomotion. The function of tendon is determined by the biochemical composition and macromolecular structural organization of its extracellular matrix (ECM), which consists of a dense network of cross-linked type I collagen with smaller amounts of type III collagen, elastin and various proteoglycans (22). This collagen-rich tissue provides structural support to the tendon, selectively binds and releases growth factors that regulate multiple cellular functions, and organizes the compartments that contain various cell populations (5). Tendon fibroblasts are the predominant cell type in tendon, and are responsible for the production, maintenance, modification and repair of matrix proteins (22). Despite the importance of tendon to the overall function of the musculoskeletal system, relatively little is known about the cellular and molecular mechanisms that regulate tendon growth and remodeling in the adult.

Tendon adapts to increased mechanical loads by undergoing hypertrophy, as evidenced by increases in tendon cross-sectional area (CSA), cell density, peak stress, peak strain and type I collagen content (9, 27, 43). While the specific growth factors and signaling pathways required for this process are largely unknown, one family of growth factors that are upregulated following tendon injury and are active at multiple stages of

the healing process are the platelet-derived growth factors (PDGFs) (29, 55). PDGFs typically function as secreted hetero- or homodimers of disulfide-linked polypeptide chains (PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and PDGF-AB) (1, 12, 14), with PDGF-BB among the most frequent PDGF isoforms used to enhance regeneration in various animal models of tendon injury (23, 54). PDGFs bind to and activate a class of structurally related receptor tyrosine kinase transmembrane proteins known as PDGF receptors α (PDGFRα) and β (PDGFRβ) (1, 12, 14). Upon binding to their ligands, PDGFRs dimerize (αα, αβ and ββ) and undergo autophosphorylation of conserved cytoplasmic tyrosine residues that initiate multiple signal transduction cascades, including the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and extracellular signal-related kinases 1 and 2 (ERK1/2) pathways (14). PDGFs are potent mitogens and their signaling events control key biological functions in many cell types of mesenchymal origin, including proliferation, differentiation, migration and ECM synthesis and remodeling (1, 14, 46). While PDGFs are expressed in tendon tissue, the identity and tissue localization of PDGFRα^+ and PDGFRβ^+ cells in tendon have not been clearly defined, and the overall importance of PDGFR signaling to the growth response of mechanically loaded tendons is presently unknown.

During periods of tendon growth and remodeling, fibroblasts express matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that collectively degrade multiple ECM components, including type I collagen (5, 43). Membrane-type MMPs (MT-MMPs) are a sub-class of MMPs that allow cells to migrate through their respective ECMs, by constraining type I collagen degradation to the leading edge of the cell membrane (8, 16). Within the MT-MMP subgroup, membrane type-1 MMP (MT1-

MMP) is required for proper tendon development, as the deletion of MT1-MMP in embryonic tendon fibroblasts results in the failure of normal tendon formation (51). MT1-MMP expression has also been shown to correlate with pivotal events in the growth response of mechanically-loaded tendons of adult animals (43). In other cell types, MT1-MMP expression is regulated by receptor tyrosine kinase signaling, (13, 48), but the signal transduction pathways that regulate MT1-MMP expression in tendon fibroblasts are not known.

Given that PDGFs are expressed in tendon, and that MT1-MMP is important for tendon development, we sought to determine the role of PDGFR signaling in postnatal tendon growth and remodeling. We hypothesized that inhibition of PDGFR signaling would suppress the normal growth of tendon tissue during mechanical overload due to defects in cell proliferation and migration. To test this hypothesis, we induced tendon growth in adult mice via mechanical overload using the hindlimb synergist ablation model, and blocked PDGFRα and PDGFRβ signaling through the use of a pharmacological inhibitor. We also conducted a series of *in vitro* experiments to determine the molecular mechanisms that underlie the PDGFR-dependent growth of tendons in adult animals.

Methods

Mice. All animal studies were approved by the University of Michigan Institutional Animal Care & Use Committee. Wild-type (WT) C57BL/6 mice and transgenic *PDGFRα*$^{eGFP/+}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME,

USA). *PDGFRα*$^{eGFP/+}$ mice express the H2B-eGFP reporter gene from the endogenous PDGFRa locus (10). Four-month-old male mice were used in all experiments.

Synergist Ablation. Mice were randomized to 3- and 10-day groups. Bilateral synergist ablation procedures were performed under isoflurane anesthesia as described previously (9, 46). An overview is presented in Figure 1A. The Achilles tendon was surgically excised to prevent the gastrocnemius and soleus muscles from plantarflexing the talocrural joint, resulting in compensatory hypertrophy of the synergist plantaris musculotendinous unit. Buprenorphine was administered for post-operative analgesia, and *ad libitum* weight-bearing and cage activity were allowed in the postoperative period. Mice were closely monitored during the postoperative period for any adverse reactions. At tissue harvest, the left plantaris tendons were collected for gene expression analysis, while the right plantaris tendons were used for histological examination. After the tendons were removed, mice were euthanized by cervical dislocation and induction of bilateral pneumothorax. Plantaris tendons from additional non-overloaded mice were obtained as described above for gene expression analysis.

Inhibition of PDGFR Signaling. Within each group of mice, half of the mice were treated with vehicle or CP-673,451 (Biorbyt, Cambridge, UK), a specific inhibitor of PDGFRα and PDGFRβ (6, 38). CP-673,451 inhibits both PDGFRα and PDGFRβ kinases with greater than 450-fold selectivity compared to other structurally related kinases, including vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF1R) (38). CP-673,451 was dissolved first in 2 parts dimethyl sulfoxide and then in 8 parts phosphate-buffered saline. CP-673,451 was

administered by intraperitoneal injection at 15 mg/kg twice daily, with a total daily dose of 30 mg/kg, starting the day prior to synergist ablation and continued each day until tissue harvest. This dose has previously been shown to be effective at inhibiting PDGFR phosphorylation in mice *in vivo* (38, 46).

Histology. Histological examination of the tendon tissue was performed as described previously (43, 46). Plantaris tendons were placed in 30% sucrose solution for one hour, snap frozen in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA, USA) and stored at -80°C until use. Tendons were sectioned at a thickness of 10 µm in a cryostat and stained with hematoxylin and eosin (H&E) to determine tendon CSA and cell density. For immunohistochemistry, tendon sections were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% goat serum. To identify cell types in non-overloaded and overloaded tendons, slides from *PDGFR*$\alpha^{EGFP/+}$ mice were incubated with rabbit anti-PDGFRβ (1:100; sc-339, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies. To identify proliferating cells in overloaded tendons treated with vehicle or PDGFR inhibitor, slides from WT mice subjected to synergist ablation were incubated with rabbit anti-Ki67 (1:100; ab16667, Abcam, Cambridge, MA, USA) primary antibodies. Primary rabbit antibodies against p-PDGFRα^{Y849}/PDGFRβ^{Y857} (1:100; #3170, Cell Signaling Technology, Danvers, MA, USA) were also used on slides from C57BL/6 mice to determine differences in the level of PDGFR phosphorylation in response to vehicle or PDGFR inhibitor treatment. The tendon ECM was identified with wheat germ agglutinin (WGA) lectin conjugated to Alexa Fluor 488 (AF488) (1:200; W11261, Thermo Fisher Scientific, Carlsbad, CA, USA). Secondary antibodies conjugated to AF555 (1:300; A-21429,

Thermo Fisher Scientific) were used to detect primary antibodies. Nuclei were counterstained with DAPI (1:500; D9542, Sigma Aldrich, St. Louis, MO, USA). High-resolution digital images were captured with an Olympus BX-51 microscope and camera (Olympus, Center Valley, PA, USA) for the H&E and Ki67 slides, while a Nikon A1 confocal laser microscrope (Nikon Instruments, Tokyo, JP) was used for the PDGFR slides. Quantification was performed by study personnel in a blinded fashion using ImageJ software (NIH, Bethesda, MD, USA).

Cell Culture. Fibroblasts were isolated from the tail tendons of mice as described previously (17). Tail tendons are useful for obtaining a large number of early passage cells, and they arise from the same population of somitic progenitor cells as limb tendons during development (31). Briefly, mice were anesthetized as described above, the tail was removed, and animals were euthanized by cervical dislocation. Fascicles were isolated from tail tendons, and then were finely minced and placed in low-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) containing 0.2% type II collagenase (Gibco) for 1 hour at 37°C with constant agitation. An equal volume of growth medium (GM) that consists of low-glucose DMEM with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic (Gibco) was added to the digested tissue to inactivate the collagenase. Cells were pelleted by centrifugation at 300*g* for 10 minutes, resuspended in GM and plated on 100-mm type I collagen-coated dishes (Corning, Corning, NY, USA). All cells were maintained in humidified incubators at 37°C and 5% CO_2. Passage 2-3 cells were used in all experiments. To block PDGFR signaling, cells were incubated with 1 μM CP-673,451, as described above.

Migration Assay. Type I collagen was acid-extracted from cadaver rat tail tendons as described by Hotary et al (16). Briefly, tendons were excised and placed in 0.2% acetic acid for 5 days at 4°C. The collagen solution was then centrifuged at 24,000*g* for 30 minutes, and the supernatant was collected, lyophilized and dissolved again in 0.2% acetic acid to a final concentration of 2.7 mg/ml. Collagen hydrogels were prepared in the upper chambers of Transwell dishes (12-mm diameter, 3-μm pore size; Corning) by combining the collagen solution with 10× Minimum Essential Medium (MEM, Thermo Fisher Scientific) and 0.34 N NaOH in an 8:1:1 ratio. After 45 minutes at 37°C, the gelling process was complete and 1×10^5 tendon fibroblasts were seeded on top of the collagen hydrogels. PDGF-BB (20 ng/ml; R&D Systems, Minneapolis, MN, USA) was added to the lower chambers to initiate migration of the cells into the collagen hydrogels. Where indicated, media was supplemented with TGF-β1 (10 ng/ml; PeproTech, Rocky Hill, NJ, USA), the synthetic broad spectrum MMP inhibitor BB-94 (5 μM; Tocris Bioscience, Bristol, UK), the mitogen-activated protein kinase kinases 1 and 2 (MEK1/2) inhibitor PD98059 (50 μM; InvivoGen, San Diego, CA, USA) or the PI3K inhibitor wortmannin (10 μM; InvivoGen). All media, including growth factors and inhibitors, were replaced every 2 days. Migratory activity was monitored by phase-contrast microscopy, and the cells were allowed to migrate for 6 days. The number of migrating cells and the maximum distance migrated were quantified in 5 randomly selected fields of a single experiment from 3 or more independent experiments performed.

Proliferation Assay. Uptake of bromodeoxyuridine (BrdU) by proliferating tendon fibroblasts was measured as described previously (28). Tendon fibroblasts were

incubated overnight with or without 20 ng/ml of PDGF-BB in media containing 0.5% FBS. After overnight incubation, fresh media was added along with 20 μM of BrdU (Sigma Aldrich) for 1 hour. Cells were then rinsed with phosphate-buffered saline, fixed in ice-cold methanol and permeabilized with 0.5% Triton X-100. The BrdU epitope was exposed by denaturing DNA with 2 M HCl. BrdU was visualized with an anti-BrdU antibody (1:50; G3G4, Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) and a secondary antibody conjugated to AF555 (1:200; A-21127, Thermo Fisher Scientific). Nuclei were counterstained with DAPI (1:500; D9542, Sigma Aldrich) to determine total cell number. The number of proliferating cells were quantified in 5 randomly selected fields of a single experiment from 6 independent experiments performed.

Quantitative RT-PCR. Gene expression analysis was performed as described previously (17, 47). Plantaris tendons were homogenized in QIAzol (Qiagen, Valencia, CA, USA) and RNA was purified using a miRNeasy Micro Kit (Qiagen) supplemented with DNase I (Qiagen). RNA was reverse transcribed into cDNA with iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA). Amplification of cDNA was performed in a CFX96 real-time thermal cycler (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad). Target gene expression was normalized to the stable housekeeping gene peptidylprolyl isomerase D (PPID), and further normalized to tendons that were not subjected to synergist ablation using the $2^{-\Delta\Delta Ct}$ method. PPID was selected as a housekeeping gene from microarray data and validated with qPCR. For cell culture experiments, relative copy number was calculated using the linear regression of efficiency method (40). Primer sequences are provided in Table S1.

Microarray. Microarray measurements were performed by the University of Michigan DNA Sequencing Core as described previously (17, 46). Equal amounts of RNA isolated from four individual tendons were pooled into a single sample for microarray analysis, and two pooled samples from each group were analyzed. RNA was pooled because gene expression from a pooled sample is similar to the average of the individual samples composing the pooled sample (3, 21). Biotinylated cDNA was prepared using the GeneChip WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA, USA) and hybridized to Mouse Gene 2.1 ST Array Strips (Affymetrix). Raw microarray data were loaded into ArrayStar version 12.1 (DNASTAR, Madison, WI, USA) to calculate fold changes in gene expression. The microarray dataset was made available through the NIH Gene Expression Omnibus database (accession number GSE95794).

siRNA Transfection. Predesigned fluorescent-labeled siRNAs directed against mouse MT1-MMP (NM_008608; SI02733822, Qiagen) were transfected into tendon fibroblasts using Lipofectamine RNAiMAX (Thermo Fisher Scientific) at a final concentration of 10 nM. AllStars Negative Control siRNA (Qiagen) was used as a negative control. Fluorescence microscopy demonstrated that ~99% of the cells were transfected at 24 hours. Efficiency of MT1-MMP mRNA knockdown was determined 48 hours after transfection by qPCR.

Immunoblots. Immunoblots were performed as described (17, 46). Whole tendons and cell pellets were homogenized in ice cold RIPA buffer (Sigma Aldrich) supplemented with 1% protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein homogenates were diluted in Laemmli's sample buffer, boiled for two minutes and 20 μg of protein was separated on either 6% or 12% SDS-PAGE gels

depending on the protein of interest. Proteins were transferred to 0.45-μm nitrocellulose membranes (Bio-Rad) using the Trans-Blot SD semi-dry transfer apparatus (Bio-Rad), blocked with 5% non-fat powdered milk in TBST solution and incubated with primary rabbit antibodies (1:1000; Cell Signaling Technology) against p-PDGFRα^{Y849}/PDGFRβ^{Y857} (#3170), PDGFRα (#3174), PDGFRβ (#3169), p-ERK1/2$^{T202/Y204}$ (#4370), ERK1/2 (#4695), p-Akt^{T308} (#13038), Akt (#4691), p-p70S6K^{T389} (#9234) and p70S6K (#2708), primary rabbit antibodies (1:1000; Abcam) against MT1-MMP (ab51074) or primary rabbit antibodies (1:1000; Santa Cruz Biotechnology) against procollagen type I (Pro-Col1a1; sc-30136). β-tubulin (ab6046, Abcam) or Coomassie staining were used to determine equal protein loading. Following primary antibody incubation, membranes were rinsed and incubated with HRP-conjugated goat anti-rabbit secondary antibodies (1:10,000; ab97051, Abcam). Proteins were detected using enhanced chemiluminescent reagents (Bio-Rad) and visualized using a digital chemiluminescent documentation system (Bio-Rad).

Cell Surface Biotinylation. Cell surface proteins from tendon fibroblasts were biotinylated and purified using the Cell Surface Protein Isolation Kit (Thermo Fisher Scientific). Briefly, cells in monolayer were washed with ice-cold phosphate-buffered saline and incubated with 0.25 mg/ml of Sulfo-NHS-SS-Biotin for 30 minutes at 4°C with constant agitation. Adherent cells were then lysed and the biotinylated proteins were affinity-purified using streptavidin agarose beads. Biotinylated proteins were analyzed by immunoblotting with anti-MT1-MMP (ab51074, Abcam) antibodies. GAPDH (MA5-15738, Thermo Fisher Scientific) from the whole cell lysate and Coomassie staining were used to determine equal protein loading.

Statistics. Results are presented as mean±SD. Prism version 7.0 (GraphPad Software, La Jolla, CA, USA) was used to conduct statistical analyses. A two-way ANOVA (α=0.05) followed by Tukey's post hoc sorting evaluated the interaction between time after synergist ablation and PDGFR inhibitor treatment. For cell culture experiments, differences between groups were tested with an unpaired Student's t-test or a one-way ANOVA followed by Tukey's post hoc sorting where indicated with α=0.05.

Results

PDGFRα and PDGFRβ are expressed in tendon. We first sought to determine which cells in tendon express the PDGFRs. Tendon fibroblasts in non-overloaded tendons express both PDGFRα and PDGFRβ ($PDGFR\alpha^+/PDGFR\beta^+$) (Figure 3.1B). This was also observed within regions of neotendon tissue in the 3- and 10-day overloaded tendons (Figure 3.1C-D). Very few cells were found that express PDGFRα alone ($PDGFR\alpha^+/PDGFR\beta^-$), while a small population of cells near blood vessels in the epitenon of non-overloaded tendons and in the neotendon of overloaded tendons were discovered to express PDGFRβ alone ($PDGFR\alpha^-/PDGFR\beta^+$). *In vitro*, tendon fibroblasts expressed both PDGFR subtypes ($PDGFR\alpha^+/PDGFR\beta^+$) (Figure 3.1E).

PDGFR inhibition prevents growth of adult tendon tissue after mechanical overload. To determine the functional importance of PDGFR signaling during postnatal tendon growth, pharmacological blockade of both PDGFR subtypes was performed in adult wild-type C57BL/6 mice followed by mechanical overload using the hindlimb synergist ablation model. To verify the ability of CP-673,451 to inhibit phosphorylation of both PDGFR subtypes in tendon fibroblasts, cells isolated from tail tendons of wild-type

C57BL/6 mice were growth arrested in serum-free media and incubated alone or with PDGF-BB for 30 minutes. Under these conditions, tendon fibroblasts responded to PDGF-BB stimulation by activating PDGFRα and PDGFRβ (Figure 3.2A). In contrast, tendon fibroblasts pretreated with 1 μM CP-673,451 displayed no activation of the PDGFR subtypes upon stimulation with PDGF-BB (Figure 3.2A). Next, we collected whole tendon lysates from 3-day overloaded mice that had been treated with vehicle or CP-673,451 to determine the ability of CP-673,451 to inhibit phosphorylation of both PDGFR subytpes *in vivo*. Mice treated with CP-673,451 demonstrated reduced levels of p-PDGFRα^{Y849}/PDGFRβ^{Y857} compared to vehicle-treated controls (Figure 3.2B). Additionally, mice treated with the PDGFR inhibitor displayed significant attenuation of major downstream signaling cascades as evidenced by reduced levels of p-Akt^{T308} and p-ERK1/2$^{T202/Y204}$ compared to vehicle-treated controls (Figure 3.2B). These findings were also supported by immunofluorescence of tendon sections taken 3 days after mechanical overload, which demonstrated fewer cells expressing p-PDGFRα^{Y849}/PDGFRβ^{Y857} in response to PDGFR inhibitor treatment (Figure 3.2C).

Mechanical overload of the plantaris tendons resulted in outward growth of a neotendon matrix from the most superficial layers of the original tendon (Figure 3.3A). Overall, PDGFR inhibition did not impact the general morphological features of the plantaris tendons, but noticeable differences in tendon CSA and cell density were observed (Figure 3.3B-G). For the original tendon, the CSA of the 10-day vehicle group was slightly larger than both the 3-day vehicle and PDGFR inhibitor groups, but otherwise CSA of the original tendon did not change in response to time after overload or treatment with PDGFR inhibitor (Figure 3.3B). In contrast, the CSA of the neotendon

in the 3- and 10-day PDGFR inhibitor groups was 50% and 59% smaller compared to their respective vehicle-treated controls (Figure 3.3C). Changes in total tendon CSA generally followed the same trends observed in the neotendon data (Figure 3.3D). For the original tendon, the cell density of all groups was similar and did not change in response to time after overload or treatment with PDGFR inhibitor (Figure 3.3E). However, the cell density of the neotendon in the 10-day vehicle group was 60% higher compared to both the 3-day vehicle and PDGFR inhibitor groups, and this increase was not observed in response to PDGFR inhibition (Figure 3.3F). Similar to the total tendon CSA results, changes in total tendon cell density generally followed the same trends observed in the neotendon data (Figure 3.3G).

Previous reports have demonstrated PDGF-BB-dependent proliferation of tendon fibroblasts *in vitro* (53), and while we also observed that tendon fibroblasts stimulated with PDGF-BB proliferate at higher rates compared to cells cultured in low-serum media (Figure 3.4), we were mainly interested in determining if global inhibition of PDGFR signaling during mechanical overload prevents proliferation of tendon fibroblasts. In general, mechanical overload results in greater abundance of Ki67-expressing cells in the neotendon compared to the original tendon of all groups (Figure 3.5A). For the original tendon, the number of proliferating cells in all groups was similar and did not change in response to time after overload or treatment with PDGFR inhibitor (Figure 3.5B). In contrast, the number of proliferating cells in the neotendon of the 3- and 10-day PDGFR inhibitor groups was 55% and 76% less compared to their respective vehicle-treated controls (Figure 3.5C). Changes in the number of proliferating cells in the total tendon generally followed the same trends observed in the neotendon data

(Figure 3.5D). Taken together, these results indicate that PDGFR inhibition suppresses the normal growth of tendon tissue after mechanical overload, and this is partly explained by defects in cell proliferation.

Transcriptomic analysis of PDGFR-inhibited tendons demonstrates defects in angiogenesis, ECM synthesis and remodeling, expression of MMPs and tissue inhibitors of metalloproteinases (*TIMPs), cell specification and proliferation, and cell migration.* Since inhibition of PDGFR signaling resulted in a tendon growth phenotype, we sought to further explore the mechanisms behind this response by conducting transcriptional profiling. Using a minimum and maximum fold change of 1.5, microarray experiments identified that approximately 10% of the genome was differentially expressed in both the 3- and 10-day groups, with slight differences between vehicle- and PDGFR-treated animals at each time point (Figure 3.6A-B). By 10 days, the overall number of differentially expressed transcripts was less than 3 days, although the number of unique transcripts expressed in both PDGFR inhibitor groups was similar at each time point (Figure 3.6B). Gene ontology (GO) analysis demonstrated that PDGFR signaling governs multiple processes critical to tendon fibroblast function, with the greatest enrichment observed in biological adhesion and locomotion (Figure 3.6C).

Based on the results of the microarray experiments and GO analysis, we followed up on genes of interest with qPCR. For markers of angiogenesis, multiple genes demonstrated reduced expression in the 10-day PDGFR inhibitor compared to vehicle-treated controls, including platelet/endothelial cell adhesion molecule 1 (PECAM1), also known as CD31, which is a membrane glycoprotein involved in cell adhesion of endothelial cells, and CD146, another adhesion molecule that is highly

expressed by pericytes (Figure 3.7A). CD248, which is a C-type lectin domain protein important for angiogenesis, was did not increase at 3 and 10 days in response to PDGFR inhibition. Interestingly, thrombospondin 1 (TSP1), which is a secreted glycoprotein that inhibits angiogenesis through direct effects on endothelial cell proliferation and migration, also did not increase at 10 days in the PDGFR inhibitor group compared to vehicle-treated controls, while slight changes in the expression of the angiogenic growth factor vascular endothelial growth factor A (VEGFA) were noted as a result of time after overload. In addition to the changes observed in markers of angiogenesis, numerous ECM synthesis and remodeling genes were also affected by PDGFR inhibition (Figure 3.7B). Expression of all the fibrillar types I, III and V collagens as well as the network types IV and VI collagens did not increase at 10 days in response to PDGFR inhibition, while no differences in expression for any of the collagens were observed between the 3-day vehicle and PDGFR inhibitor groups. Given that type I collagen is the main structural protein of tendon, we sought to determine if the reduction in type I collagen transcripts at the whole tissue level was a direct effect of PDGFR inhibition on tendon fibroblasts or an indirect effect related to a decrease in cell number. Tendon fibroblasts were isolated from tail tendons of wild-type C57BL/6 mice and incubated alone or with PDGF-BB for 24 hours in serum-free conditions. PDGF-BB stimulation had no significant effect on Col1a1 and Col1a2 transcript levels or Pro-Col1a1 protein expression (Figure 3.8A-C). These results indicate that PDGF-BB does not regulate expression of type I collagen at the mRNA and protein levels in tendon fibroblasts, and hence the reduction in type I collagen transcripts at the whole tissue level is likely an indirect effect related to a decrease in cell number.

Accompanying the changes observed in ECM synthesis and remodeling genes, PDGFR inhibition also resulted in marked changes in the expression of MMPs and their endogenous TIMPs (Figure 3.7C). For the gelatinases, MMP2 expression was 59% lower at 10 days in the PDGFR inhibitor group compared to vehicle-treated controls, while MMP9 was 83% higher at 3 days in response to PDGFR inhibition. Similar to MMP9, the collagenase MMP8 was 89% higher at 3 days in response to PDGFR inhibition, whereas no differences in the expression of collagenase MMP13 were demonstrated in response to time after overload or treatment with PDGFR inhibitor. MT1-MMP, also known as MMP14, was dramatically upregulated by greater than 5- and 46-fold at 3 and 10 days after mechanical overload, respectively, and treatment with the PDGFR inhibitor prevented the increase in transcript levels at 10 days compared to vehicle-treated controls. Compared to the 3-day time point, TIMP1 transcript levels were lower in both 10-day groups, while TIMP2 transcript levels did not increase at 10 days in response to PDGFR inhibition.

Transcription factors that play crucial roles in different stages of tendon development were upregulated after mechanical overload (Figure 3.7D). The basic helix-loop-helix transcription factor scleraxis (Scx) was upregulated greater than 2-fold at both 3 and 10 days and was not affected by PDGFR inhibitor treatment. After an initial downregulation of the atypical homeodomain transcription factor mohawk (Mkx) and the type II transmembrane glycoprotein tenomodulin (Tnmd) at 3 days, their transcript levels increased by 10 days compared to non-overloaded controls in the absence of PDGFR inhibition. The zinc finger transcription factors early growth response 1 and 2 (Egr1 and Egr2) did not increase at 10 days in response to PDGFR

inhibition, while Egr2 expression was also 71% lower at 3 days in the PDGFR inhibitor group compared to vehicle-treated controls. The changes in Ki67 signal present on immunofluorescence (Figure 3.5A) were accompanied by quantitative changes in gene expression. Marker of proliferation Ki67 (Mki67) transcript levels did not increase at 3 and 10 days in response to PDGFR inhibition. In addition to the changes observed in cell specification and proliferation genes, numerous cell migration genes were also affected by PDGFR inhibition (Figure 3.7E). Angiomotin-like protein 2 (AmotL2), which is a membrane-associated scaffold protein that localizes to lamellipodia during cell migration, and myosin light chain 9 (Myl9), which regulates contractility and cytoskeletal tension during cell migration, both did not increase at 10 days in response to PDGFR inhibition. The cytokinetic scaffold protein anillin (Anln), which binds F-actin and can recruit Rho GTPases to the leading edge of the cell membrane during cell migration, did not increase at 3 days in response to PDGFR inhibition. Other actin-binding proteins that help reorganize the actin cytoskeleton during cell migration include transgelin (Tagln), filamin A (Flna) and diaphanous related formin 3 (Diaph3), and whereas the expression of all of these genes increased after mechanical overload, none of them were affected by PDGFR inhibitor treatment. In contrast, ankyrin repeat domain 1 (Ankrd1), which is a transcriptional repressor of MMP13, increased by greater than 3-fold in the 3-day PDGFR inhibitor group compared to vehicle-treated controls. The matricellular proteins cysteine-rich angiogenic inducer 61 (Cyr61) and connective tissue growth factor (CTGF) are key mediators of cell migration through their interaction with cell surface integrins, and while Cyr61 was not affected by PDGFR inhibition, CTGF expression was 65% lower at 10 days in the PDGFR inhibitor group compared to

vehicle-treated controls. Overall, these results indicate that PDGFR inhibition exerts profound effects on the transcriptome of plantaris tendons during mechanical overload, and influences multiple processes critical to proper tendon growth, including angiogenesis, ECM synthesis and remodeling, proliferation and cell migration.

MT1-MMP is an essential proteinase for tendon fibroblast migration through tendon ECM. Based on the gene expression data, we identified MT1-MMP as the most highly upregulated MMP after mechanical overload whose expression at 10 days was attenuated in response to PDGFR inhibition (Figure 3.7C). Previous reports have recognized MT1-MMP as a key effector of migratory behavior in many different cell types due to its pericellular collagenolytic activity (8, 41), but its role in tendon fibroblast migration has remained largely unexplored. Using cross-linked type I collagen hydrogels that closely recapitulate the tendon ECM environment *in vivo* (16, 41), tendon fibroblasts were cultured on top of the hydrogels and migration was initiated by a chemotactic gradient of PDGF-BB (Figure 3.9A). Over a 6-day culture period, tendon fibroblasts migrated into the hydrogel and the number of migrating cells as well as the maximum distance migrated were assessed by light microscopy (Figure 3.9B-D). In the absence of PDGF-BB, or in response to transforming growth factor beta 1 (TFG-β1) treatment, which has been shown to stimulate tendon fibroblast migration in explant cultures of rat patellar tendon (7), tendon fibroblasts did not migrate into the hydrogel. Furthermore, the migratory activity of tendon fibroblasts was completely inhibited in the presence of BB-94, a synthetic broad spectrum MMP inhibitor, which indicates that the ability of tendon fibroblasts to migrate through the hydrogel is a proteinase-dependent process. To determine the role of MT1-MMP in tendon fibroblast migration, siRNAs

were directed against MT1-MMP and their impact on the migratory activity of tendon fibroblasts was assessed. Silencing efficiency of the MT1-MMP siRNAs was confirmed without detectable off-target effects on other MMP transcripts (Figure 3.8D-E). Over a 6-day culture period, the chemotactic response of MT1-MMP-silenced tendon fibroblasts to PDGF-BB stimulation was completely abolished compared to scrambled siRNA controls (Figure 3.10A-C). Taken together, these results indicate that MT1-MMP is required for tendon fibroblast migration through cross-linked type I collagen hydrogels that closely recapitulate tendon ECM environment *in vivo*.

PDGF-BB increases MT1-MMP expression in tendon fibroblasts via PI3K/Akt-mediated translational and ERK1/2-mediated post-translational mechanisms. Activation of the PI3K/Akt and ERK1/2 pathways has been previously linked to PDGF-BB-dependent migratory activities in other cell types (48). To determine the integrity of these downstream signaling cascades in tendon fibroblasts, cells isolated from tail tendons of wild-type C57BL/6 mice were growth arrested in serum-free media and incubated alone or with PDGF-BB for 60 minutes. Under these conditions, tendon fibroblasts responded to PDGF-BB stimulation by activating both Akt and ERK1/2 (Figure 3.11A). Both maximum Akt and ERK1/2 activation occurred at 5 minutes, and while p-Akt^{T308} levels then steadily declined, p-ERK1/2$^{T202/Y204}$ levels were more transient and returned to near baseline levels by 60 minutes post-stimulation. Consistent with Akt activation, p-p70S6K^{T389} levels began to rise within 5 minutes and peaked at 15 minutes with sustained activation noted thereafter. To gain insight into the regulation of tendon fibroblast migration by the PI3K/Akt and ERK1/2 pathways, cells were cultured on top of collagen hydrogels and migration was assessed in the presence

of either the PI3K inhibitor wortmannin or the MEK1/2 inhibitor PD98059. Over a 6-day culture period, the migratory activity of tendon fibroblasts in the presence of either inhibitor was significantly inhibited as assessed by light microscopy (Figure 3.11B-D). Next, we sought to determine the molecular connection between MT1-MMP expression and both the PI3K/Akt and ERK1/2 pathways in tendon fibroblasts. Cells isolated from tail tendons of wild-type C57BL/6 mice were incubated alone or with PDGF-BB for 24 hours in low-serum conditions. PDGF-BB stimulation had no significant effect on MT1-MMP transcript levels (Figure 3.12A). On immunoblots, three distinct bands were visualized for the MT1-MMP protein at 63, 60 and 45 kD, which correspond to the pro-form, the active form and the processed form of the enzyme (Figure 3.12B). In response to PDGF-BB stimulation, Akt and ERK1/2 activation were accompanied by a 1.4-fold increase in total MT1-MMP protein expression, which includes all forms of the enzyme (Figure 3.12C-E). However, in the presence of wortmannin, activation of Akt was blocked, but total MT1-MMP protein expression did not increase. In the presence of PD98059, while the activation of ERK1/2 was blocked, total MT1-MMP protein expression increased similar to PDGF-BB stimulation in the absence of either inhibitor. Interestingly, the increase in total MT1-MMP protein expression in the PD98059 group was accounted for by an increase in the pro-form of the enzyme, while the active and processed forms did not increase (Figure 3.12F-H). Thus, the ratio between the active and pro-forms of MT1-MMP was 47% lower in the PD98059 group compared to all other groups (Figure 3.12I). Given that the active form of MT1-MMP can be found at the cell membrane where it is responsible for extracellular proteolysis, we next sought to determine cell surface levels of the MT1-MMP protein in response to PDGF-BB

treatment. Cells isolated from tail tendons of wild-type C57BL/6 mice were incubated alone or with PDGF-BB for 24 hours in low-serum conditions, after which cell surface biotinylation and immunoblotting were performed. Similar to the findings from whole cell lysates, an increase in cell surface MT1-MMP was demonstrated with PDGF-BB stimulation, while both wortmannin and PD98059 blocked the increase of MT1-MMP at the cell surface (Figure 3.13A-C). Take together, these results indicate that PDGF-BB-dependent MT1-MMP expression in tendon fibroblasts is regulated by PI3K/Akt-mediated translational and ERK1/2-mediated post-translational mechanisms (Figure 3.13D).

Discussion

The ability of tendon tissue to transmit force generated by muscular contraction is critical to the overall health of the musculoskeletal system. In response to changes in mechanical load, tendon actively remodels its ECM to meet the new demands placed on it, which is typically accompanied by increases in tendon CSA, cell density and type I collagen content (9, 27). While PDGFR signaling is known to regulate fundamental biological activities of many cells that compose musculoskeletal tissues, its role in postnatal tendon growth and remodeling has remained largely unexplored. In the current study, we report that tendon fibroblasts express both PDGFRs, PDGFRα and PDGFRβ, and that inhibition of signaling through these receptors during mechanical overload suppresses the normal growth of tendon tissue due to defects in cell proliferation and migration. Furthermore, we identify MT1-MMP as an essential proteinase for tendon fibroblast migration through tendon ECM, and demonstrate that

MT1-MMP translation is regulated by PI3K/Akt, while ERK1/2 controls post-translational trafficking of MT1-MMP within tendon fibroblasts. These findings highlight the crucial role that PDGFR signaling plays in the adaptation of adult tendons to mechanical growth stimuli, and earmark MT1-MMP as a potential therapeutic target in the treatment of tendon injuries and diseases.

The growth of new tendon tissue during mechanical loading is a complex process that requires a coordinated effort between tendon fibroblasts and their local ECM environment. Tendon fibroblasts constantly sense and respond to biomechanical and biochemical cues in the tendon ECM, and an increase in mechanical load placed on the tendon is often a signal for growth. In response to a mechanical growth stimulus, tendon fibroblasts begin to proliferate, migrate into areas of newly formed tendon tissue and synthesize new matrix proteins. Each of these elements – proliferation, migration and ECM synthesis and remodeling – are required for proper tendon growth, and a defect in any one of them can result in impairment of this process. In the current study, we demonstrated that growth of the plantaris tendon from mechanical overload was prevented by PDGFR inhibition, largely due to defects in cell proliferation and migration. Our findings of smaller tendons with fewer proliferating cells in PDGFR inhibitor-treated animals is supported by PDGF-BB-dependent increases in proliferation and migration of tendon fibroblasts in our tissue culture experiments. Additionally, differences in tendon morphology as a result of PDGFR inhibition were accompanied by quantitative changes in the expression of cell proliferation and migration genes. Mki67 expression did not increase at 3 and 10 days in response to PDGFR inhibition, and various genes involved in actin reorganization and cytoskeletal tension, including AmotL2, Myl9 and Anln, also

did not increase in the PDGFR inhibitor-treated animals during mechanical overload. Interestingly, Ankrd1 was significantly upregulated in response to PDGFR inhibition, whereas transcript levels of the matricellular protein CTGF did not increase. CTGF has been shown to enhance cell migration by upregulating MMP13 expression, while Ankrd1 is a known transcriptional repressor of MMP13 (50). Even though MMP13 expression was not directly affected by PDGFR inhibition, mechanical overload resulted in MMP13 upregulation compared to non-overloaded controls. As tendons grow in response to mechanical loading, they require ingrowth of new vasculature to keep up with the metabolic demands of the tissue. Even though we did not detect any gross morphological changes in capillary structure, the transcript levels of multiple angiogenesis genes failed to increase due to PDGFR inhibition, including the endothelial cell adhesion molecules PECAM1 and CD248, as well as the pericyte marker CD146, which represents a potential source of pluripotent stem cells during tendon regeneration (19, 24). Finally, the expression of TSP1, an endogenous inhibitor of angiogenesis, did not increase in the overloaded PDGFR inhibitor-treated mice, and TSP1 has been shown to activate latent transforming growth factor beta (TGF-β) to enhance ECM synthesis and remodeling (49).

Mechanical overload resulted in upregulation of many mesenchymal tendon fibroblast markers, including Scx, Tnmd, Mkx, Egr1 and Egr2, which is consistent with previous studies of tendon growth (27, 43). PDGF-BB is known to regulate Egr1 transcript levels, and both Egr1 and Egr2 expression in PDGFR inhibitor-treated mice were blocked during mechanical overload compared to vehicle-treated controls (30). Many of these transcription factors are known to regulate type I collagen expression by

tendon fibroblasts (26), and PDGFR inhibition in the current study produced significant changes in ECM synthesis and remodeling genes. For all of the fibrillar (I, III and V) and network (IV and VI) collagens, increases in their transcript levels at 10 days were blocked in the PDGFR inhibitor-treated mice compared to vehicle-treated controls. However, we looked at type I collagen expression more closely by tendon fibroblasts *in vitro*, since it is the predominant structural protein in tendon, and found that tendon fibroblasts treated with PDGF-BB demonstrated no significant increases in both Col1a1 and Col1a2 transcript levels or Pro-Col1a1 protein levels. These results indicate that type I collagen expression by tendon fibroblasts is not transcriptionally or translationally regulated by PDGF-BB, and further suggests that the decrease in type I collagen expression observed at the whole tissue level was likely an indirect effect from reduced cell number, or it was mediated through an intermediary signaling molecule or growth factor other than PDGF-BB. Along these lines, previous studies have demonstrated increased type I collagen synthesis in canine tendon fibroblasts and explant cultures of rabbit tendons as a result of PDGF-BB stimulation, but this was measured as ^{3}H-proline incorporation without measuring transcript or protein levels (53, 56). Finally, mechanical overload resulted in upregulation of multiple MMPs, including MMP2, MMP8, MMP9, MMP13 and MT1-MMP, as well as their endogenous inhibitors TIMP1 and TIMP2. These findings are consistent with previous models of tendon growth (27, 43), and while PDGFR inhibition demonstrated mixed effects on the expression of the various metalloproteinases, these results are representative of the dramatic remodeling that takes place in the tendon ECM during mechanical overload.

In mechanically-loaded tendons, adaptation of the tendon ECM is mediated by fibroblasts migrating into tendon fascicles and synthesizing new matrix proteins (27). The tendon ECM is highly enriched in type I collagen, a protein whose triple helical structure makes it resistant to most forms of proteolytic attack (33). For tendon fibroblast migration to occur, type I collagen and other matrix proteins need to be cleaved in order to create space for the cells to move. In the current study, we identify MT1-MMP as the essential proteinase for tendon fibroblast migration through cross-linked type I collagen hydrogels that closely recapitulate the tendon ECM environment *in vivo*. While secreted MMPs play an important role in tissue remodeling, their site of action is distant to their point of secretion, which impairs their ability to regulate cell migration (41, 42). In contrast, MT-MMPs are anchored to the cell membrane, which confines their type I collagenolytic activity to the pericellular microenvironment, and supports their role as the primary effectors of cellular movement (16, 41). Our findings are consistent with previous studies that have shown cells deficient in MT1-MMP lose their ability to penetrate type I collagen barriers (18, 41, 45). Knockout mouse models of single MMPs have also been generated to test their importance *in vivo*, with most mice being viable and fertile with very subtle developmental defects, except for mice lacking MT1-MMP (32). These mice die within the first few weeks of life and display significant defects in fat, bone, cartilage, skeletal muscle and tendon, suggesting that MT1-MMP plays critical roles in the growth and development of mesenchymal tissues (15, 51). Additionally, mice lacking MT1-MMP in tendon fibroblasts (ScxCre:MT1-MMP$^{f/f}$) demonstrate a severe limb phenotype with marked dorsiflexion of the fore and hind paws (51), providing further evidence for the importance of MT1-MMP to overall tendon fibroblast

function. Apart from its role in cell migration, MT1-MMP also regulates other key cellular processes including proliferation, differentiation and cell survival, by either changing the tissue architecture or converting matrix proteins into signaling molecules (18, 32).

Unlike other musculoskeletal tissues, the molecular signaling pathways that play important roles during tendon growth and remodeling in the adult have yet to be defined. While the TGF-β superfamily has been shown to be essential for the embryonic formation of tendons (11, 37), very few studies have investigated which signaling pathways are activated in response to mechanical loading in the adult, and of those activated, which are the most important for tendon growth. Popov and colleagues (36) demonstrated that mechanically stretching tendon progenitor/stem cells (TPSCs) in culture at 8% strain led to activation of ERK1/2 and p38 mitogen-activated protein kinase (MAPK), but not Akt. Interestingly, increased MT1-MMP mRNA and protein levels were also reported in this model, although the mechanism for this upregulation was not determined. Although p38 MAPK is activated in response to mechanical stimulation *in vitro,* inhibition of p38 MAPK had a negligible impact on tendon growth *in vivo* after hindlimb synergist ablation (43). In another study, local insulin-like growth factor 1 (IGF-1) expression and ERK1/2 activation was demonstrated in rat supraspinatus tendons after 16 weeks of downhill treadmill running (44). However, this model caused degenerative changes in the tendon ECM and is more commonly used to study tendinopathy than tendon growth. To our knowledge, the current study is the first to demonstrate that a particular class of cell surface receptors, namely PDGFRα and PDGFRβ, are necessary for tendon growth *in vivo*. Inhibition of PDGFR signaling *in vivo* blocked phosphorylation of important regulatory sites on Akt and ERK1/2. The T308 site

on Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1), while the T202/Y204 sites on ERK1/2 are phosphorylated by MEK1/2 (25). One of the main downstream targets of Akt is p70 S6 kinase (p70S6K), which upon mechanistic target of rapamycin complex 1 (mTORC1)-mediated phosphorylation at the T389 site, it can then activate several proteins involved in ribosomal biogenesis and the initiation of mRNA translation (57). In contrast, activated ERK1/2 regulates the activities of several transcription factors via phosphorylation that lead to the expression of genes involved in proliferation, differentiation and cell migration (1). Upon ligand-induced PDGFR autophosphorylation, both the PI3K/Akt and ERK1/2 pathways through their respective transcriptional targets have been shown to regulate the growth of a wide variety of cell types and tissues (1). In the current study, the changes in phosphorylation of Akt and ERK1/2 in the PDGFR inhibitor-treated animals correlate with the observed changes in tendon growth, and while PDGFR inhibition of perivascular cells may affect angiogenesis, most of the observed changes in tendon growth are likely the result of a direct effect of the PDGFR inhibitor on tendon fibroblasts, given that they are the predominant cell type in adult tendon tissue.

Activation of the PI3K/Akt and ERK1/2 pathways in mechanically-loaded tendons is consistent with PDGF-BB-dependent activation of these pathways in tendon fibroblasts *in vitro*. Both the PI3K/Akt and ERK1/2 pathways mediate tendon fibroblast migration through type I collagen hydrogels, and inhibition of these pathways results in decreased migratory activity of the cells similar to the MT1-MMP-silenced tendon fibroblasts. A previous study by Sun and colleagues (48) demonstrated that rat bone marrow-derived mesenchymal stem cells treated with PDGF-BB increased MT1-MMP

expression at the mRNA and protein levels, and this response was mediated by the PI3K/Akt and ERK1/2 pathways. The results from the current study do not support these findings, which suggests that the regulation of MT1-MMP expression in response to PDGF-BB stimulation is cell type and tissue dependent. Tendon fibroblasts treated with PDGF-BB produce more MT1-MMP protein, but without a change in MT1-MMP transcript levels. Treatment with the PI3K inhibitor wortmannin blocked the increase in MT1-MMP protein, which indicates that MT1-MMP mRNA translation is regulated by the PI3K/Akt pathway in a PDGF-BB-dependent manner. However, treatment with the MEK1/2 inhibitor PD98059 did not block the increase in total MT1-MMP protein, but did block the increase in the active form of the enzyme. Interestingly, the increase in total MT1-MMP protein in the presence of PD98059 is accounted for by an increase in the pro-form of the enzyme. To our knowledge, the mechanism by which ERK1/2 inhibition leads to more of the pro-form compared to the active form of the enzyme is not known. MT1-MMP is synthesized as a zymogen and requires processing in the trans-Golgi network (TGN) by furin, a proprotein convertase that recognizes and cleaves a basic motif in the prodomain of MT1-MMP, which then exposes the catalytic domain and allows the enzyme to become functional (18). Furin is a type I transmembrane protein that functions as a serine endoprotease and belongs to the subtilisin-like proprotein convertase (SPC) family (52). Interestingly, some members of the SPC family are known to be regulated by PDGF-BB (2), and furin expression itself has been shown to be regulated by ERK1/2 activation (35). Furthermore, furin can be phosphorylated in its cytoplasmic tail by casein kinase II which controls its trafficking to the TGN (20). In the current study, ERK1/2 inhibition results in an increase in the pro-form of MT1-MMP,

which could be explained by a defect in its processing to the active form of the enzyme. Our findings indicate that additional studies are needed to further investigate the role of ERK1/2 inhibition on the trafficking of furin to the TGN. Finally, when cells are stimulated by growth factors such as PDGF-BB, they can quickly mobilize intracellular stores of MT1-MMP to the cell surface, often to specialized membrane protusions known as lamellipodia or invadopodia where ECM degradation occurs (34). Previous studies have shown this process is regulated by both the PI3K/Akt and ERK1/2 pathways, and our results from this study are consistent with those findings.

Although we demonstrated the importance of PDGFR signaling during postnatal tendon growth and remodeling, there are several limitations to the current study. We used a specific inhibitor of PDGFRα and PDGFRβ, and while the off-target effects of this inhibitor were minimized due to its affinity for the PDGFRs compared to other structurally related kinases, we were not able to selectively inhibit each receptor to determine its individual function (38). It also remains possible that some of the observed changes in tendon growth were due to the effect of the PDGFR inhibitor on the plantaris muscle (46). However, even if PDGFR inhibition resulted in less force production by the overloaded plantaris muscle, we would still expect the plantaris tendon to be loaded during the muscle-independent phase of gait. Whereas more than 20 MMPs have been described, we only silenced MT1-MMP during our migration assays, thus it remains possible that other MMPs are also important for tendon fibroblast migration (32). Additionally, we measured differences in tendon morphology after mechanical overload due to PDGFR inhibition, but tendon mechanics and other functional assays were not performed. The synergist ablation procedure is reliable, reproducible and can be simply

executed, but the mechanical load placed on the plantaris tendon is greater than that experienced during normal locomotion. Furthermore, while our study focused only on the plantaris tendon, observed differences might not be reflected in other trunk or limb tendons. We measured the expression of many transcripts using microarray and qPCR, but changes in the transcriptome might not follow changes in the proteome. Finally, we collected data at only two time points after mechanical overload, and perhaps longer term experiments may provide additional mechanistic insight.

In summary, this is the first study to demonstrate that a family of RTKs, namely PDGFRα and PDGFRβ, and their ligands, the PDGFs, are necessary for proper tendon growth and remodeling in the adult. As with all physiological processes, normal tendon function is the result of a complex interplay between the cells that make up the tissue and their local ECM environment. While we demonstrate that PDGFR signaling is a key regulator of tendon fibroblast proliferation and migration, it is interesting to note that dysfunctional PDGFR activity has been linked to a hypercellular phenotype that is commonly seen in painful tendinopathies (39), and that PDGFs are upregulated and active at multiple stages of the healing process in injured tendons (29, 55). Knowledge of PDGF-dependent processes has led to the development of newer technologies such as PDGF-BB-coated sutures, which are currently being tested for clinical use in lacerated flexor tendons (4). However, further studies are needed that selectively inactivate each PDGFR subtype in order to parse out their individual functions, and provide additional mechanistic insight into the role of PDGFR signaling in tendon tissue. Given the limited treatment options available for tendon injuries and diseases, a better understanding of PDGFR signaling and the fundamental cellular and molecular

mechanisms that it regulates during tendon growth will help inform regenerative strategies for these debilitating conditions.

Acknowledgements

The authors would like to acknowledge helpful discussions and technical support by Dr. Stephen J Weiss and Dr. Farideh Sabeh. This work was supported by NIH/NIAMS grants R01-AR063649 and F32-AR067086.

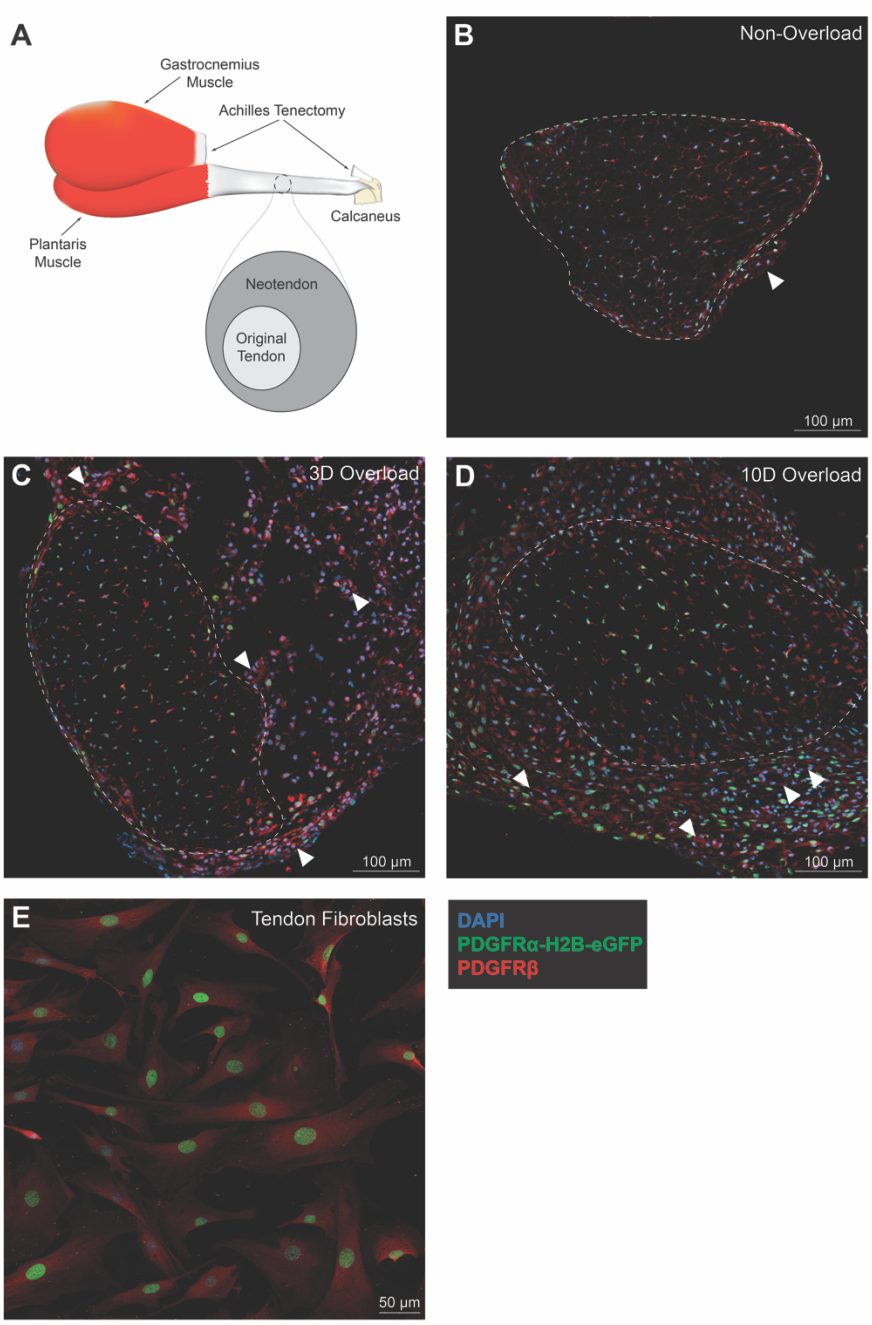

Figure 3.1. Tendon fibroblasts express both PDGFRα and PDGFRβ. (A) Schematic of the synergist ablation procedure. All histological sections were taken from the midportion of the plantaris tendon. Representative images of (B) non-overloaded, (C) 3-day overloaded and (D) 10-day overloaded plantaris tendons. Dashed white line indicates the boundary between the original tendon and neotendon. White arrowheads indicate blood vessels composed of PDGFRα-/PDGFRβ+ cells. Scale bars are 100 µm. (E) Tendon fibroblasts were isolated from PDGFRαeGFP/+ mice and immunostained for PDGFRβ. PDGFRα and PDGFRβ signals were observed in all cells. Scale bar is 50 µm. DAPI, blue; PDGFRα-H2B-eGFP, green; PDGFRβ, red.

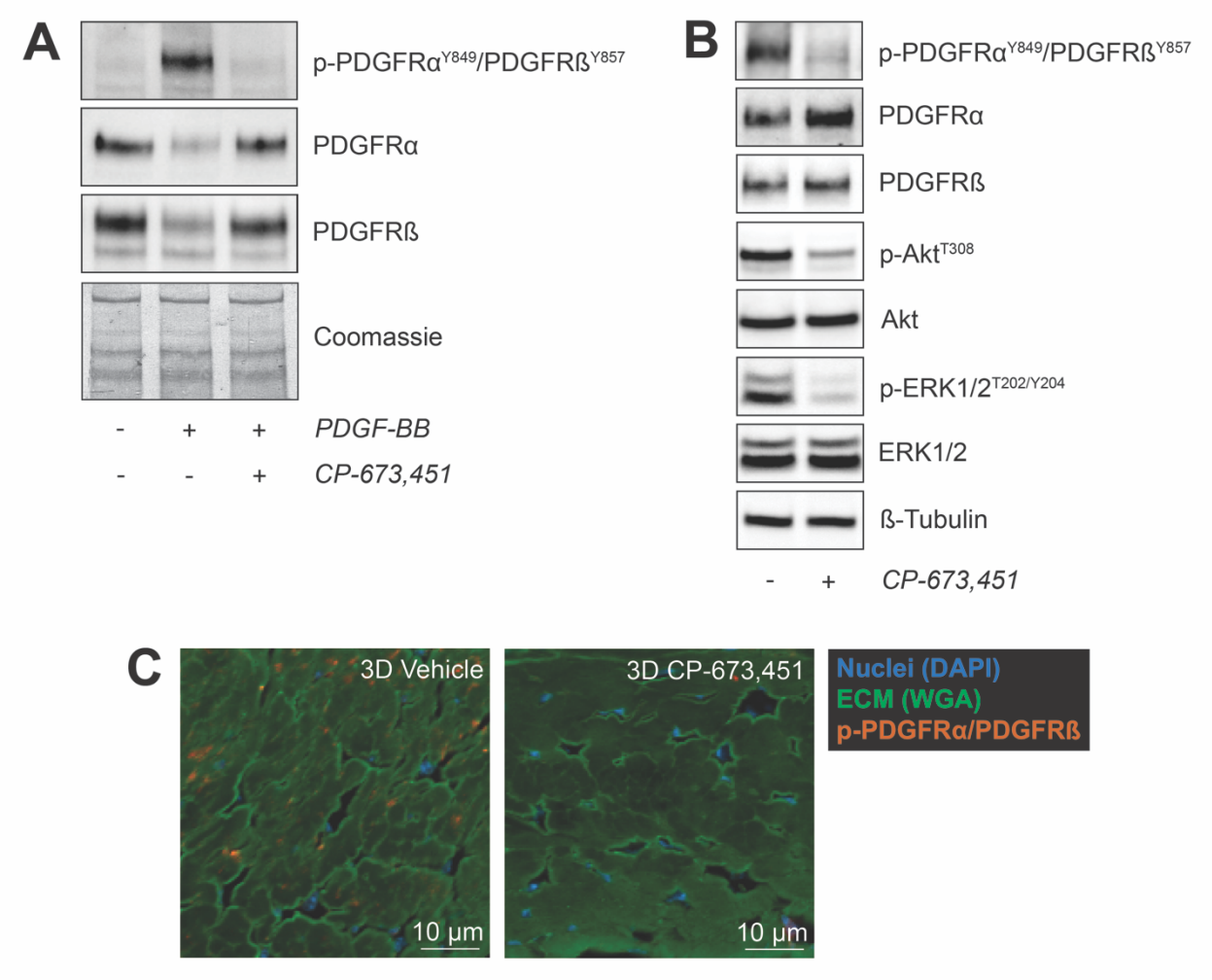

Figure 3.2. CP-673,451 inhibits phosphorylation of PDGFRα and PDGFRβ in tendon fibroblasts. (A) Representative immunoblots of serum-starved tendon fibroblasts treated with 20 ng/ml of PDGF-BB for 30 minutes with or without 1 µM of the PDGFR inhibitor CP-673,451. A Coomassie stained membrane is shown as a loading control. (B) Representative immunoblots of 3-day overloaded plantaris tendons demonstrating the ability of CP-673,451, to inhibit phosphorylation of PDGFRα and PDGFRβ in vivo. Phosphorylation of Akt and ERK1/2 were also inhibited by PDGFR inhibitor treatment. β-tubulin is shown as a loading control. (C) Immunohistochemistry of 3-day overloaded plantaris tendons treated with vehicle or PDGFR inhibitor demonstrates a decrease in the abundance of p-PDGFRα/PDGFRβ-expressing cells in the overloaded plantaris tendons treated with CP-673,451 relative to vehicle-treated controls. Scale bars are 10 µm. DAPI, blue; WGA, green; p-PDGFRαY849/PDGFRβY857, red.

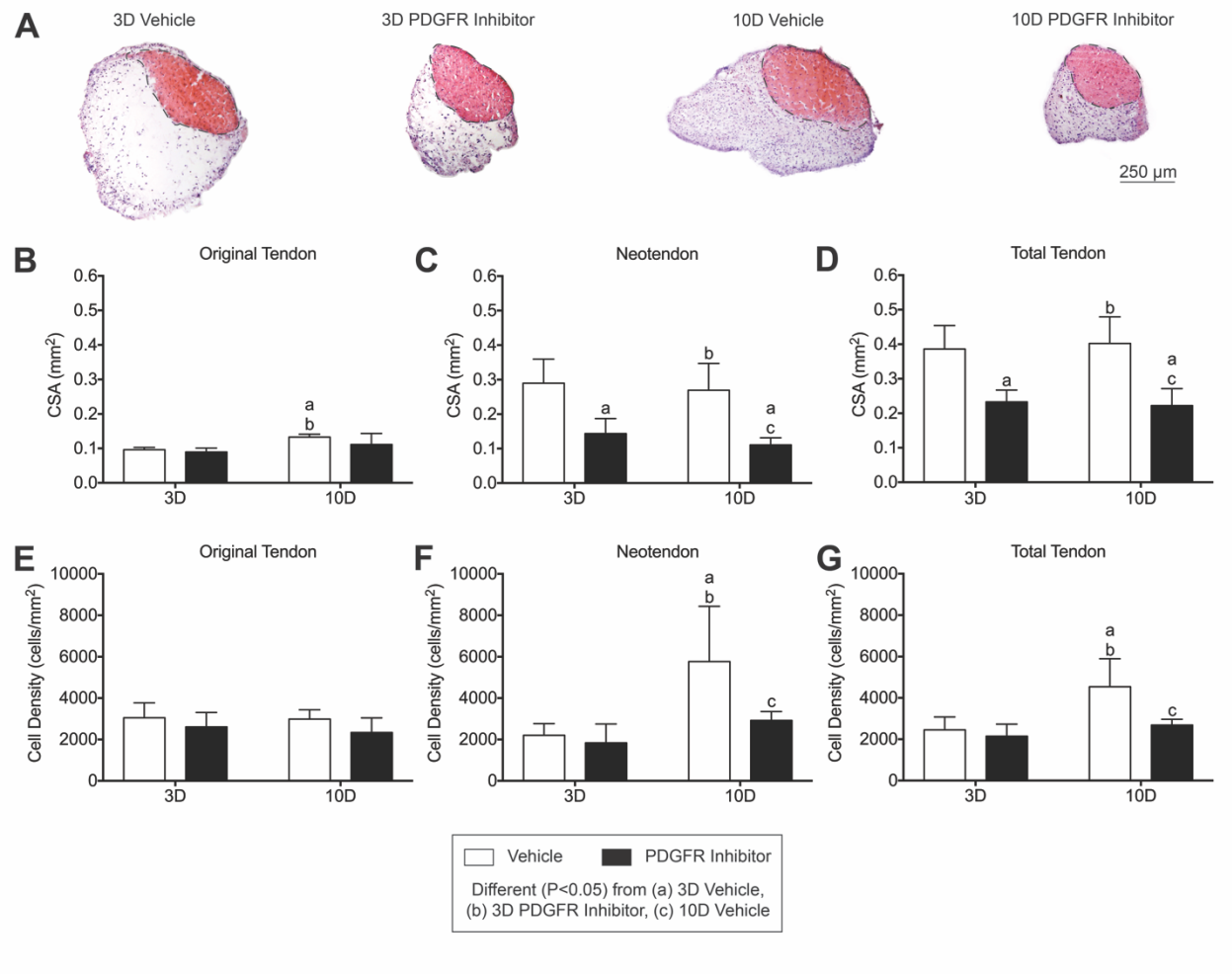

Figure 3.3. Inhibition of PDGFR signaling prevented growth of plantaris tendons subjected to mechanical overload. (A) Representative cross sections of 3- and 10-day overloaded plantaris tendons, treated with vehicle or PDGFR inhibitor, and stained with hematoxylin and eosin. Dashed black line indicates the boundary between the original tendon and neotendon. Scale bar is 250 µm. Quantitative analysis of (B-D) cross-sectional area (CSA, in mm2) and (E-G) cell density (cells/mm2) for the original tendon, neotendon and total tendon. Values are mean±SD. N=5 tendons for each group. Differences between groups were tested using a two-way ANOVA (α=0.05) followed by Tukey's post hoc sorting: different (P<0.05) from a, 3D vehicle; b, 3D PDGFR inhibitor; c, 10D vehicle.

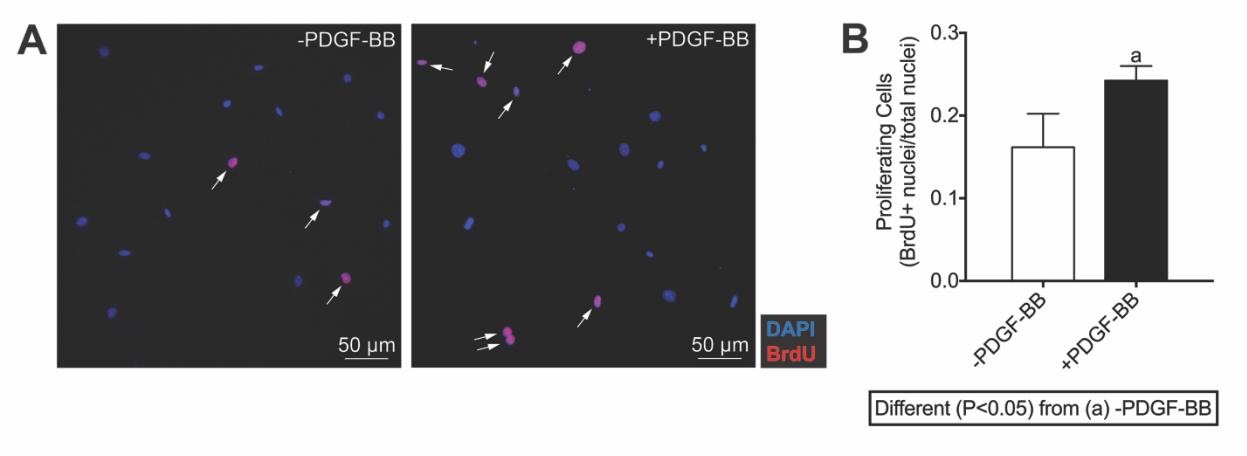

Figure 3.4. PDGF-BB stimulates proliferation of tendon fibroblasts in vitro. (A) *In vitro* proliferative activity of tendon fibroblasts was measured by BrdU uptake in the presence of 0.5% FBS with or without 20 ng/ml of PDGF-BB treatment. Proliferating cells were double labeled for DAPI (blue) and BrdU (red), and marked by white arrows. (B) Proliferative activity was quantified as mean±SD in 5 randomly selected fields of a single experiment from 6 independent experiments performed. Differences between groups were tested using an unpaired t-test; a, $P<0.05$.

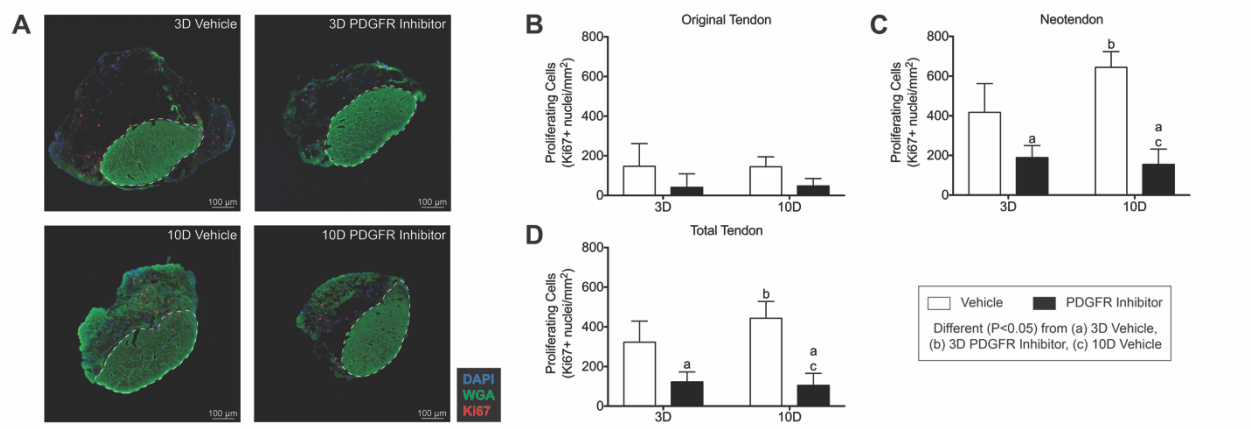

Figure 3.5. Inhibition of PDGFR signaling decreases tendon fibroblast proliferation in vivo. (A) Representative Ki67 immunostaining of 3- and 10-day overloaded plantaris tendons, treated with vehicle or PDGFR inhibitor. Dashed white line indicates the boundary between the original tendon and neotendon. Scale bars are 100 µm. DAPI, blue; WGA, green; Ki67, red. (B-D) Quantitative analysis of proliferating cells (Ki67+ nuclei/mm^2) for the original tendon, neotendon and total tendon. Values are mean±SD. N=4 tendons for each group. Differences between groups were tested using a two-way ANOVA (α=0.05) followed by Tukey's post hoc sorting: different ($P<0.05$) from a, 3D vehicle; b, 3D PDGFR inhibitor; c, 10D vehicle.

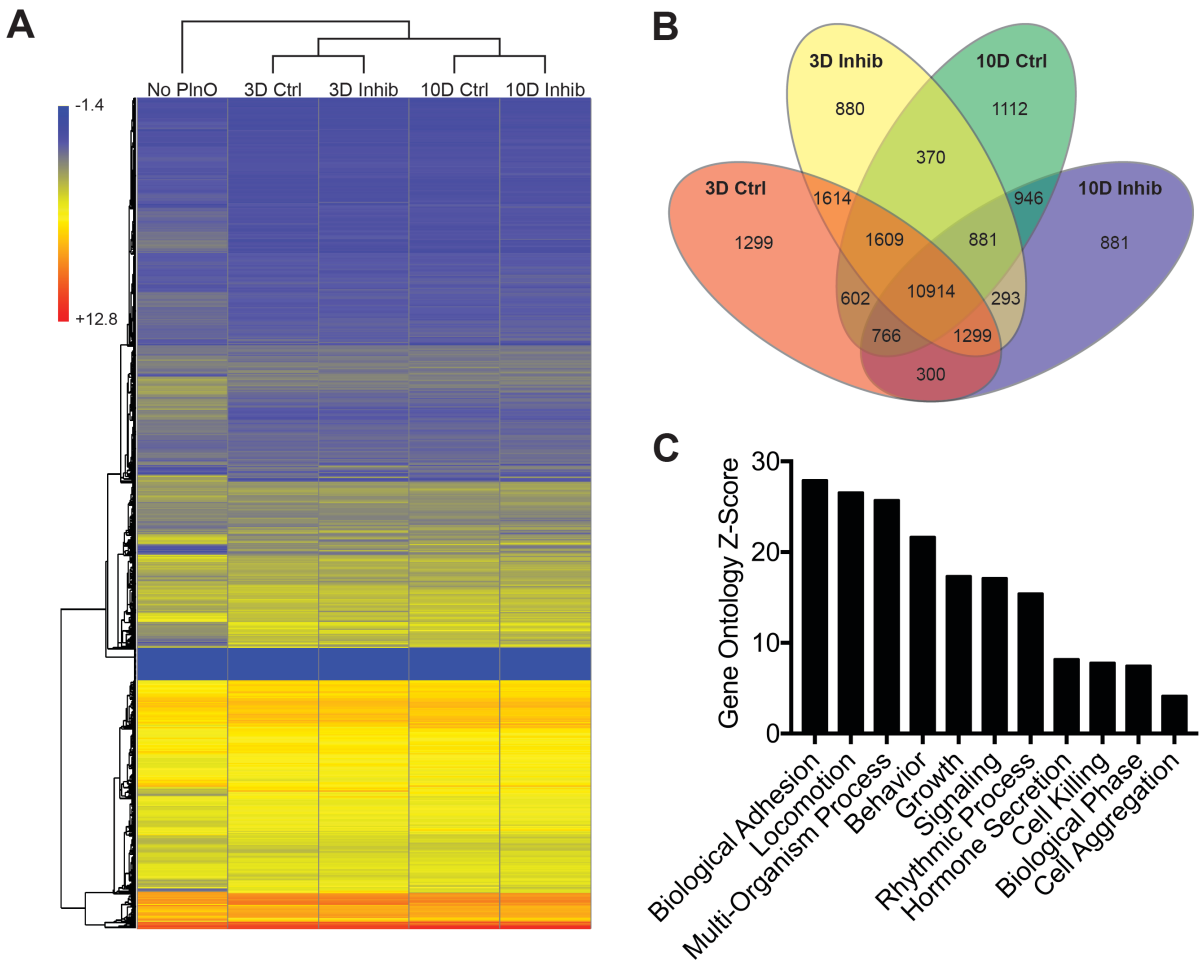

Figure 3.6. Microarray. (A) Heat map and (B) Venn diagram demonstrating the unique and overlapping transcripts that were greater than 1.5-fold upregulated or downregulated compared to non-overloaded plantaris tendons. The key to the left of the heat map assigns colors to absolute expression values of genes on a $\log_2$ scale. (C) Graphical representation of gene ontology enrichment analysis of overloaded plantaris tendons treated with PDGFR inhibitor relative to vehicle-treated controls.

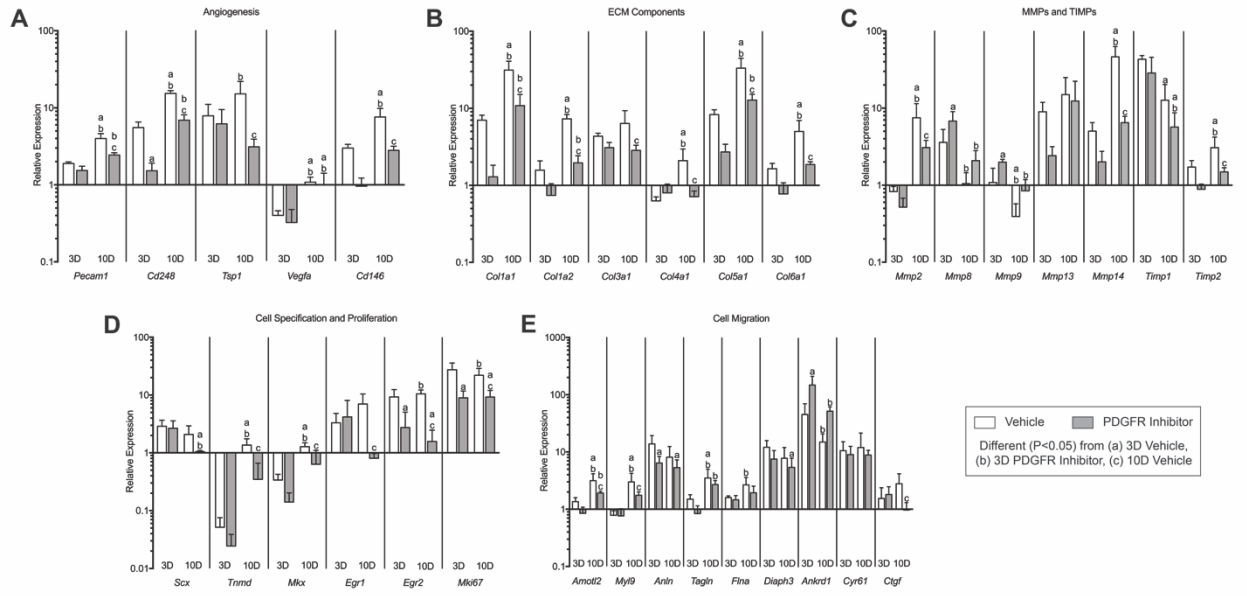

Figure 3.7. Inhibition of PDGFR signaling prevents the expression of angiogenesis, ECM synthesis and remodeling, cell specification and proliferation, and cell migration genes in plantaris tendons subjected to mechanical overload. Quantitative expression of (A) angiogenesis, (B) ECM synthesis, (C) MMPs and TIMPs, (D) cell specification and proliferation, and (E) cell migration genes in 3- and 10-day overloaded plantaris tendons, treated with vehicle or PDGFR inhibitor. Target gene expression was normalized to the stable housekeeping gene peptidylprolyl isomerase D (PPID), and further normalized to plantaris tendons that were not subjected to synergist ablation. Values are mean±SD. N=5 tendons for each group. Differences between groups were tested using a two-way ANOVA (α=0.05) followed by Tukey's post hoc sorting: different (P<0.05) from a, 3D vehicle; b, 3D PDGFR inhibitor; c, 10D vehicle.

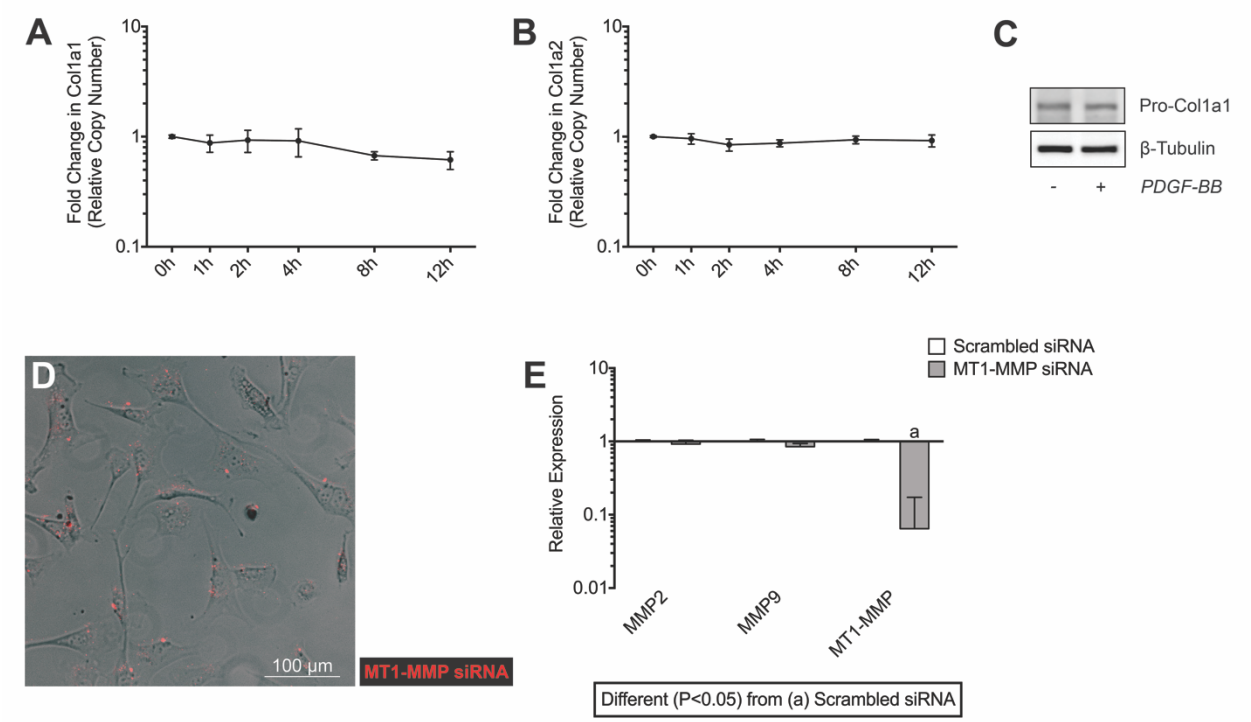

Figure 3.8. Effect of PDGF-BB treatment on collagen expression, and validation of MT1-MMP siRNA. Quantitative expression of (A) Col1a1 and (B) Col1a2 transcript levels in tendon fibroblasts treated with 20 ng/ml of PDGF-BB for 1, 2, 4, 8 and 12 hours in low-serum conditions. Values are mean±SD for 3 biological replicates. Differences between groups were tested using one-way ANOVA ($P<0.05$). No significant differences were found between groups. (C) Representative immunoblots of Pro-Col1a1 protein levels in tendon fibroblasts treated with 20 ng/ml of PDGF-BB for 24 hours in low-serum conditions. β-tubulin is shown as a loading control. (D) Representative phase contrast image of tendon fibroblasts transfected with fluorescent-labeled MT1-MMP siRNA (red) demonstrating >98% transfection efficiency. (E) Quantitative expression of MMP2, MMP9 and MT1-MMP mRNA in tendon fibroblasts transfected with scrambled or MT1-MMP siRNA for 48 hours demonstrating specificity and efficiency of MT1-MMP mRNA knockdown. Differences between scrambled siRNA and MT1-MMP siRNA for each gene were tested using t-tests; a, $P<0.05$.

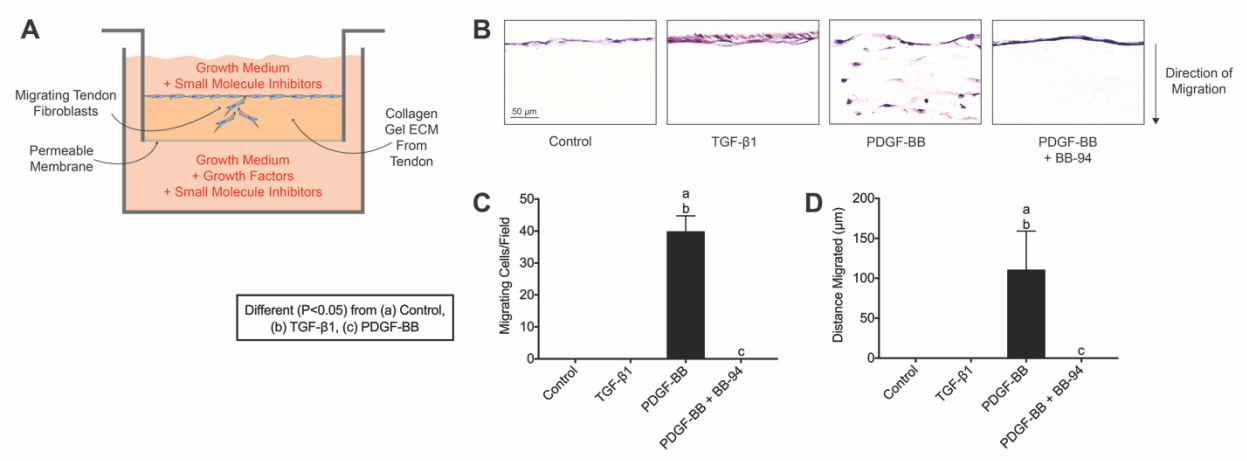

Figure 3.9. PDGF-BB stimulates tendon fibroblast migration in vitro. (A) Schematic representation of the in vitro migration assay. (B) Representative images of tendon fibroblasts obtained 6 days after being placed on the surface of a tendon ECM transwell system, treated with no additional growth factors (control), TGF-β1, PDGF-BB, and PDGF-BB + BB-94 (a synthetic broad spectrum MMP inhibitor). Quantification of (C) the number of migrating cells per field and (D) maximum distance migrated. Values are mean±SD. N=4 replicates per group. Differences between groups were tested using a one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting: different (P<0.05) from a, control; b, TFG-β1; c, PDGF-BB.

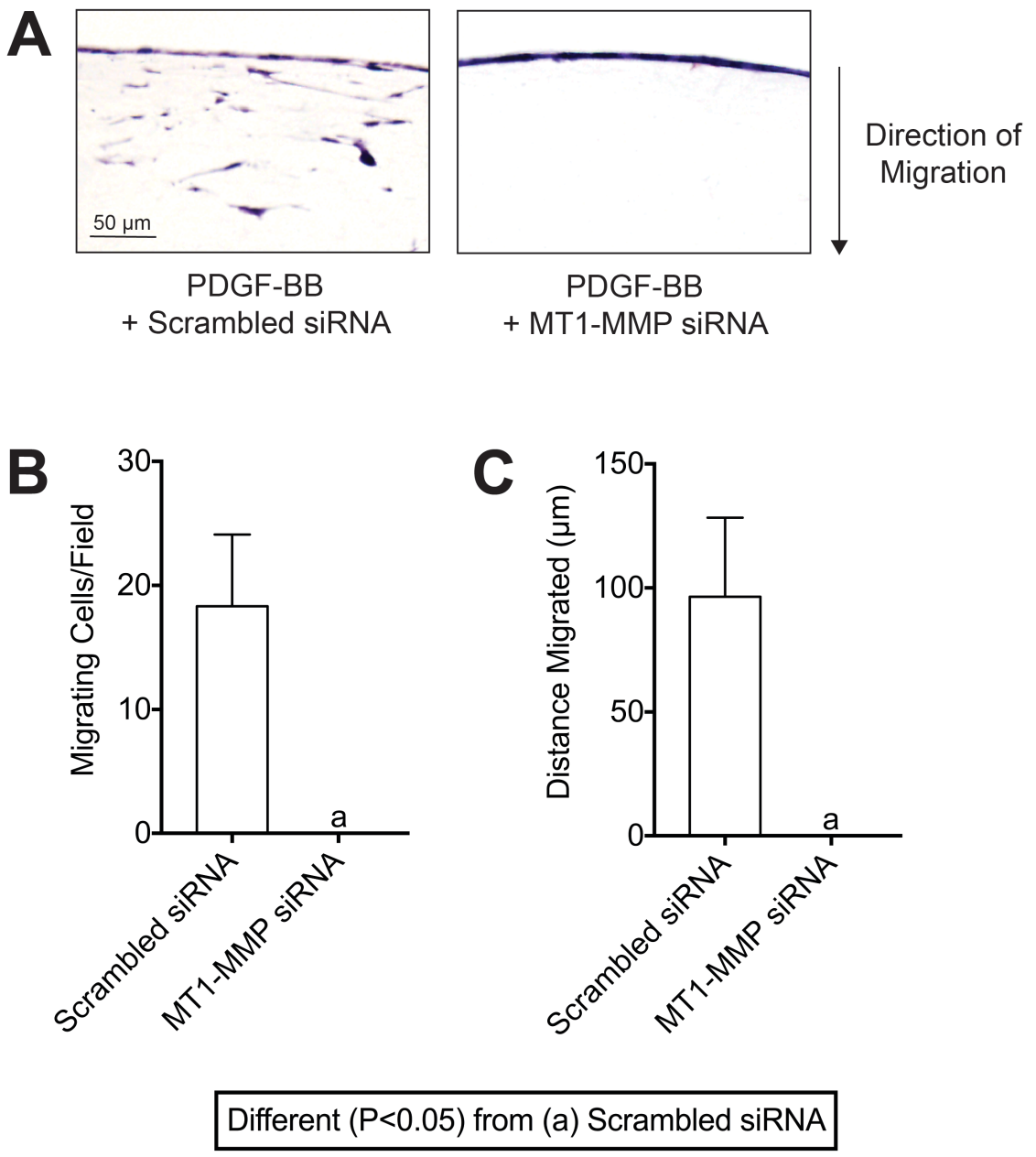

Figure 3.10. MT1-MMP is required for PDGF-BB-mediated fibroblast migration through tendon ECM. (A) Representative images of tendon fibroblasts transfected with scrambled siRNA or MT1-MMP siRNA, and treated with PDGF-BB, measured after 6 days. Quantification of (B) the number of migrating cells per field and (C) maximum distance migrated of the MT1-MMP-silenced tendon fibroblasts. Values are mean±SD. N=4 replicates per group. Differences between groups were tested using an unpaired Student's t-test; a, P<0.05.

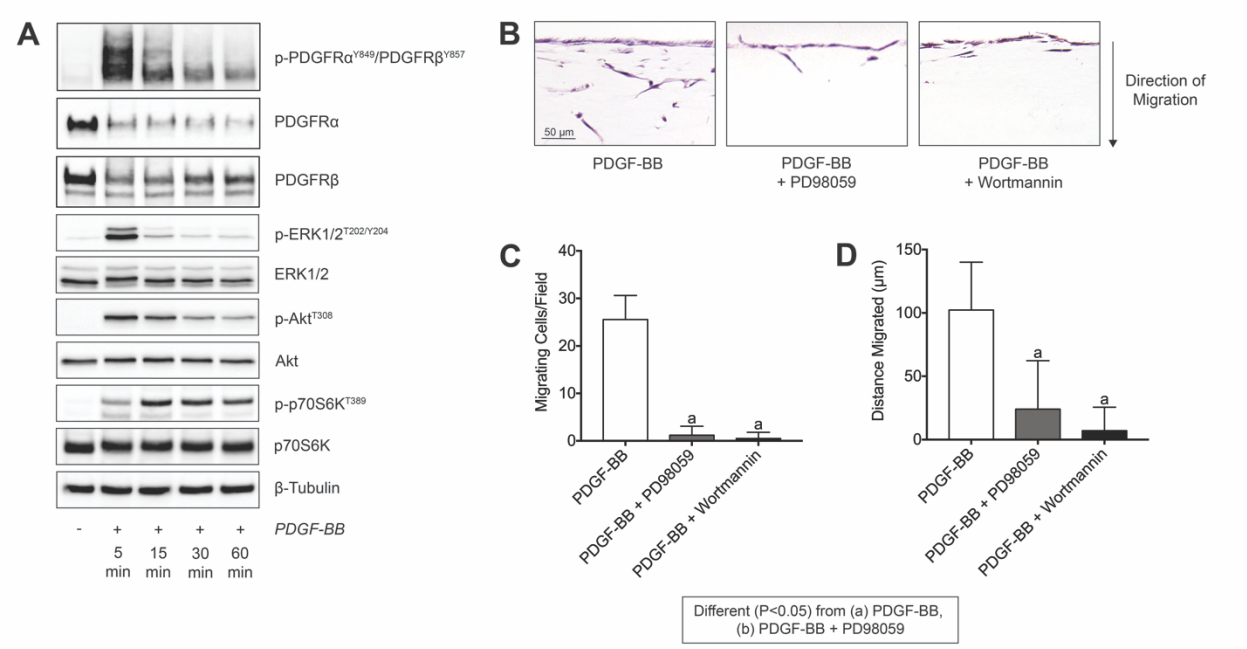

Figure 3.11. PDGF-BB stimulation of tendon fibroblasts activates PI3K/Akt and ERK1/2 pathways, which both in turn mediate tendon fibroblast migration through tendon ECM. (A) Representative immunoblots of total and phospho PDGFRα, PDGFRβ, ERK1/2, Akt and p70S6K from serum-starved tendon fibroblasts treated with PDGF-BB for 5, 15, 30 and 60 minutes. β-tubulin is shown as a loading control. (B) Representative images of tendon fibroblasts obtained 6 days after being placed on the surface of a tendon ECM transwell system, treated with PDGF-BB, PDGF-BB + PD98059, or PDGF-BB + Wortmannin. Quantification of (C) the number of migrating cells per field and (D) maximum distance migrated. Values are mean±SD. N=4 replicates per group. Differences between groups were tested using a one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting: different (P<0.05) from PDGF-BB; b, PDGF-BB + PD98059.

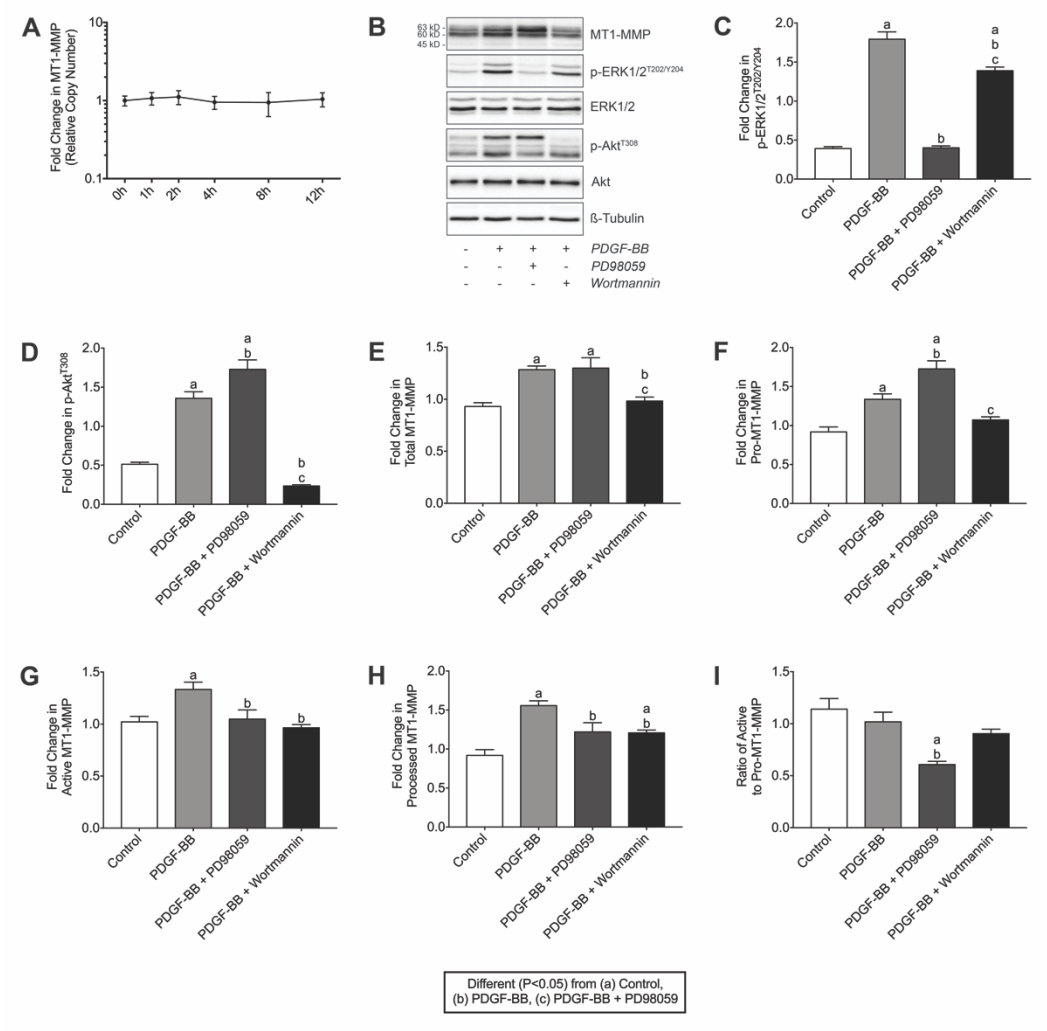

Figure 3.12. PDGF-BB does not regulate MT1-MMP mRNA abundance, but does increase MT1-MMP protein levels through a PI3/Akt-dependent mechanism. (A) Quantification of MT1-MMP transcript levels in tendon fibroblasts treated with 20 ng/ml of PDGF-BB for 1, 2, 4, 8 and 12 hours in low-serum conditions. Values are mean±SD for 3 replicates. Differences between groups were tested using one-way ANOVA ($P<0.05$). (B) Representative immunoblots of Pro-MT1-MMP (63 kD), active MT1-MMP (60 kD), processed MT1-MMP (45 kD), and phospho and total ERK1/2 and Akt are shown, from tendon fibroblasts incubated alone or with PDGF-BB in the presence or absence of PD98059 or Wortmannin for 24 hours. β-tubulin is shown as a loading control. Quantification of (C) p-ERK1/2$^{T202/Y204}$, (D) p-Akt^{T308}, (E) total MT1-MMP, (F) pro-MT1-MMP, (G) active MT1-MMP and (H) processed MT1-MMP protein levels. (I) Ratio of the pro- to active form of MT1-MMP. Values are mean±SD for 6 biological replicates. Differences between groups were tested using a one-way ANOVA ($\alpha=0.05$) followed by Tukey's post hoc sorting: a, different ($P<0.05$) from control; b, PDGF-BB; c, PDGF-BB + PD98059.

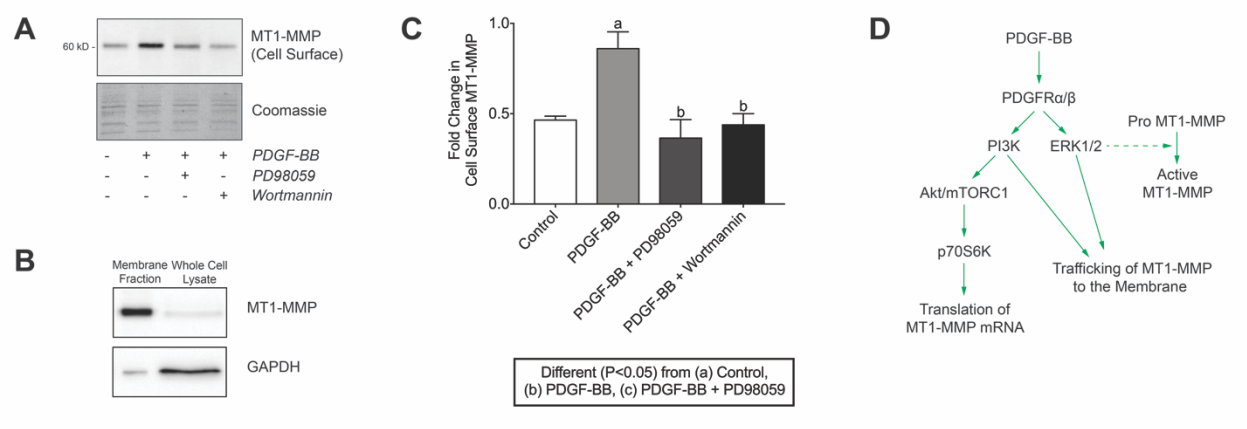

Figure 3.13. PDGF-BB-dependent membrane trafficking of MT1-MMP in tendon fibroblasts is regulated by the PI3K/Akt and ERK1/2 pathways. (A) Representative immunoblots of cell surface MT1-MMP protein levels in tendon fibroblasts after incubation alone or with PDGF-BB in the presence or absence of PD98059 or Wortmannin for 24 hours in low-serum conditions. Coomassie staining is shown as a total protein loading control. (B) Representative immunoblots of the membrane fraction and whole cell lysates of tendon fibroblasts using MT1-MMP and GAPDH as typical membrane and cytosolic proteins, respectively. Due to the isolation process, membrane fraction lanes run in a more narrow fashion. (C) Quantification of cell surface MT1-MMP protein levels. Values are mean±SD for 3 biological replicates. Differences between groups were tested using a one-way ANOVA (α=0.05) followed by Tukey's post hoc sorting: a, different ($P<0.05$) from control; b, PDGF-BB; c, PDGF-BB + PD98059. (D) Diagram of PDGF-BB-dependent regulation of MT1-MMP expression and membrane trafficking in tendon fibroblasts.

Gene	Description	Forward Primer (5'-3')	Reverse Primer (3'-5')	Size (bp)	Reference Sequence
Amotl2	Angiomotin-like 2	AACCGCCACCTGGCAAGCAA	GGTCCTCGATGGCACCACGC	148	NM_019764.2
Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	AAACGGACGGCACTCCACCG	CGCTGTGCTGAGAAGCTTGTCTCT	210	NM_013468.3
Anln	Anillin, actin binding protein	TGGGGCTGAGCAGATGGTCG	TCCGGGACTGGCCATAACTGAAGA	274	NM_028390.3
Cd146 (Mcam)	Melanoma cell adhesion molecule	GTGAATCCAACTGCCCCTGA	CCATGCAGAGATACCGTCCC	184	NM_023061.2
Cd248	CD248 antigen, endosialin	CTGTGCGTGAAACAGCCTTC	TCCACACACTCATGTTCGCA	110	NM_054042.2
Col1a1	Collagen, type I, alpha 1	ACTGCAACATGGAGACAGGTCAGA	ATCGGTCATGCTCTCTCCAAACCA	128	NM_007742.4
Col1a2	Collagen, type I, alpha 2	CCAGCGAAGAACTCATACAGC	GGACACCCCTTCTACGTTGT	105	NM_007743.2
Col3a1	Collagen, type III, alpha 1	ACGTAAGCACTGGTGGACAG	CAGGAGGGCCATAGCTGAAC	98	NM_009930.2
Col4a1	Collagen, type IV, alpha 1	GGCAGGTCAAGTTCTAGCGT	TGGCCTGATGTTGGTAACCC	106	NM_009931.2
Col5a1	Collagen, type V, alpha 1	GGAGAGCCACGTGTTCTGTAG	GAGGGAATGAGGCATGGCAG	135	NM_015734.2
Col6a1	Collagen, type VI, alpha 1	AAAGGCACCTACACCGACTG	GCATGGTTCCTTGTAGCCCT	135	NM_009933.4
Ctgf	Connective tissue growth factor	CCCTGCCCTAGCTGCCTACCG	GCTTCGCAGGGCCTGACCAT	204	NM_010217.2
Cyr61	Cysteine rich protein 61	GCCGTGGGCTGCATTCCTCT	GCGGTTCGGTGCCAAAGACAGG	262	NM_010516.2
Diaph3	Diaphanous related formin 3	GAGAAGCGACCCAAGTTGCAT	GAAGGGGAGGTCTCTCTTTCTT	109	NM_019670.1
Egr1	Early growth response 1	CAGCGCCTTCAATCCTCAAG	GCGATGTCAGAAAAGGACTCTGT	78	NM_007913.5
Egr2	Early growth response 2	GGTTGTGCGAGGAGCAAATG	GGCAGCTGGTGCATAAAACC	82	NM_010118.3
Flna	Filamin, alpha	GTACCGTGTCCGGGCTGTGC	ACATGCTCGCCACCGAAGCG	276	NM_010227.3
Mki67	Marker of proliferation Ki67	TCGTGTTACTGGCAGACGAC	ACCGCAGCTTGGTTTCTAGT	111	NM_001081117.2
Mkx	Mohawk homeobox	CAACCCGTACCCTACGAAGA	AGCCGACGTCTAGCATTAGC	102	NM_177595.4
Mmp2	Matrix metallopeptidase 2	GACCTTGACCAGAACACCATC	CATCCACGGTTTCAGGGTCC	163	NM_008610.2
Mmp8	Matrix metallopeptidase 8	TACAGGGAACCCAGCACCTA	CCACGGAGTGTGGTAGTAGC	158	NM_008611.4
Mmp9	Matrix metallopeptidase 9	AACACCACCGAGCTATCCAC	AGGAGTCTGGGGTCTGGTTT	163	NM_013599.2
Mmp13	Matrix metallopeptidase 13	CAGTTGACAGGCTCCGAGAA	TTCACCCACATCAGGCACTC	119	NM_008607.2
Mmp14	Matrix metallopeptidase 14 (membrane-inserted)	AGGCTGATTTGGCAACCATGA	CCCACCTTAGGGGTGTAATTCTG	172	NM_008608.4
Myl9	Myosin, light polypeptide 9, regulatory	AGGCCTCAGGCTTCATCCACGA	ATGGGGTCTAGGCACTGGGGC	216	NM_172118.1
Pecam 1	Platelet/endothelial cell adhesion molecule 1	GGAAGTGTCCTCCCTTGAGC	CCTTCCGTTCTTAGGGTCG	98	NM_008816.3
Ppid	Peptidylprolyl isomerase D (cyclophlin D)	AGTGAAGATGTCCCACGCAT	CCACGTCAAAGAAGACTCGC	74	NM_026352.3
Scx	Scleraxis	CCTTCTGCCTCAGCAACCAG	GGTCCAAAGTGGGGCTCTCCGTGACT	156	NM_198885.3
Tagln	Transgelin	AGGCGGCCTTTAAACCCCTCACC	CGGCCTACATCAGGGCCACAC	244	NM_011526.5
Tsp1	Thrombospondin 1	TCCAGCTCAGCTACCAAGGTC	AGGAGAGGCCACAGATAGCTT	127	NM_011580.4
Timp1	Tissue inhibitor of metalloproteinase 1	CTTGGTTCCCTGGCGTACTC	ACCTGATCCGTCCACAAACAG	150	NM_011593.2
Timp2	Tissue inhibitor of metalloproteinase 2	TCAGAGCCAAAGCAGTGAGC	GCCGTGTAGATAAACTCGATGTC	142	NM_011594.3
Tnmd	Tenomodulin	TGTACTGGATCAATCCCACTCT	GCTCATTCTGGTCAATCCCCT	114	NM_022322.2
Vegfa	Vascular endothelial growth factor A	CGTCCAACTTCTGGGCTCTT	CAGCTCCGATCGGTTTGTCT	74	NM_001025250.3

Table 3.1. Primer sequences used for quantitative PCR.

References

1. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes & Development* 22: 1276–1312, 2008.

2. Bando M, Matsuoka A, Tsuji A, Matsuda Y. The proprotein convertase PACE4 is upregulated by PDGF-BB in megakaryocytes: gene expression of PACE4 and furin is regulated differently in Dami cells. *Journal of Biochemistry* 132: 127–134, 2002.

3. Chaillou T, Jackson JR, England JH, Kirby TJ, Richards-White J, Esser KA, Dupont-Versteegden EE, McCarthy JJ. Identification of a conserved set of upregulated genes in mouse skeletal muscle hypertrophy and regrowth. *Journal of Applied Physiology* 118: 86–97, 2015.

4. Cummings SH, Grande DA, Hee CK, Kestler HK, Roden CM, Shah NV, Razzano P, Dines DM, Chahine NO, Dines JS. Effect of recombinant human platelet-derived growth factor-BB-coated sutures on Achilles tendon healing in a rat model: A histological and biomechanical study. *Journal of Tissue Engineering* 3: 1–9, 2012.

5. Davis ME, Gumucio JP, Sugg KB, Bedi A, Mendias CL. MMP inhibition as a potential method to augment the healing of skeletal muscle and tendon extracellular matrix. *J Appl Physiol* 115: 884–891, 2013.

6. Ehnman M, Missiaglia E, Folestad E, Selfe J, Strell C, Thway K, Brodin B, Pietras K, Shipley J, Östman A, Eriksson U. Distinct effects of ligand-induced PDGFRα and PDGFRβ signaling in the human rhabdomyosarcoma tumor cell and stroma cell compartments. *Cancer Research* 73: 2139–2149, 2013.

7. Fu S-C, Cheuk Y-C, Chan K-M, Hung L-K, Wong MW-N. Is cultured tendon fibroblast a good model to study tendon healing? *J Orthop Res* 26: 374–383, 2008.

8. Grant Rowe R, Keena D, Sabeh F, Willis AL, Weiss SJ. Pulmonary fibroblasts mobilize the membrane-tethered matrix metalloprotease, MT1-MMP, to destructively remodel and invade interstitial type I collagen barriers. *AJP: Lung Cellular and Molecular Physiology* 301: L683–L692, 2011.

9. Gumucio JP, Phan AC, Ruehlmann DG, Noah AC, Mendias CL. Synergist ablation induces rapid tendon growth through the synthesis of a neotendon matrix. *J Appl Physiol* 117: 1287–1291, 2014.

10. Hamilton TG, Klinghoffer RA, Corrin PD, Soriano P. Evolutionary divergence of platelet-derived growth factor alpha receptor signaling mechanisms. *Mol Cell Biol* 23: 4013–4025, 2003.

11. Havis E, Bonnin M-A, Olivera-Martinez I, Nazaret N, Ruggiu M, Weibel J, Durand

C, Guerquin M-J, Bonod-Bidaud C, Ruggiero F, Schweitzer R, Duprez D. Transcriptomic analysis of mouse limb tendon cells during development. *Development* 141: 3683–3696, 2014.

12. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiological Reviews* 79: 1283–1316, 1999.

13. Hiden U, Lassance L, Tabrizi NG, Miedl H, Tam-Amersdorfer C, Cetin I, Lang U, Desoye G. Fetal Insulin and IGF-II Contribute to Gestational Diabetes Mellitus (GDM)-Associated Up-Regulation of Membrane-Type Matrix Metalloproteinase 1 (MT1-MMP) in the Human Feto-Placental Endothelium. *The Journal of Clinical Endocrinology & Metabolism* 97: 3613–3621, 2012.

14. Hoch RV. Roles of PDGF in animal development. *Development* 130: 4769–4784, 2003.

15. Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 99: 81–92, 1999.

16. Hotary K, Allen E, Punturieri A, Yana I, Weiss SJ. Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. *J Cell Biol* 149: 1309–1323, 2000.

17. Hudgens JL, Sugg KB, Grekin JA, Gumucio JP, Bedi A, Mendias CL. Platelet-Rich Plasma Activates Proinflammatory Signaling Pathways and Induces Oxidative Stress in Tendon Fibroblasts. *Am J Sports Med* 44: 1931–1940, 2016.

18. Itoh Y. MT1-MMP: A key regulator of cell migration in tissue. *IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)* 58: 589–596, 2006.

19. James AW, Zara JN, Zhang X, Askarinam A, Goyal R, Chiang M, Yuan W, Chang L, Corselli M, Shen J, Pang S, Stoker D, Wu B, Ting K, Péault B, Soo C. Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. *Stem Cells Translational Medicine* 1: 510–519, 2012.

20. Jones BG, Thomas L, Molloy SS, Thulin CD, Fry MD, Walsh KA, Thomas G. Intracellular trafficking of furin is modulated by the phosphorylation state of a casein kinase II site in its cytoplasmic tail. *The EMBO Journal* 14: 5869–5883, 1995.

21. Kendziorski C, Irizarry RA, Chen K-S, Haag JD, Gould MN. On the utility of pooling biological samples in microarray experiments. *Proc Natl Acad Sci USA* 102: 4252–4257, 2005.

22. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiological Reviews* 84: 649–698, 2004.

23. Kovacevic D, Gulotta LV, Ying L, Ehteshami JR, Deng X-H, Rodeo SA. rhPDGF-BB Promotes Early Healing in a Rat Rotator Cuff Repair Model. *Clin Orthop Relat Res* 473: 1644–1654, 2014.

24. Lee CH, Lee FY, Tarafder S, Kao K, Jun Y, Yang G, Mao JJ. Harnessing endogenous stem/progenitor cells for tendon regeneration. *J Clin Invest* 125: 2690–2701, 2015.

25. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 141: 1117–1134, 2010.

26. Léjard V, Brideau G, Blais F, Salingcarnboriboon R, Wagner G, Roehrl MHA, Noda M, Duprez D, Houillier P, Rossert J. Scleraxis and NFATc regulate the expression of the pro-alpha1(I) collagen gene in tendon fibroblasts. *J Biol Chem* 282: 17665–17675, 2007.

27. Mendias CL, Gumucio JP, Bakhurin KI, Lynch EB, Brooks SV. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. *J Orthop Res* 30: 606–612, 2011.

28. Mendias CLC, Bakhurin KIK, Faulkner JAJ. Tendons of myostatin-deficient mice are small, brittle, and hypocellular. *Proc Natl Acad Sci USA* 105: 388–393, 2008.

29. Molloy T, Wang Y, Murrell G. The roles of growth factors in tendon and ligament healing. *Sports Med* 33: 381–394, 2003.

30. Mundschau LJ, Forman LW, Weng H, Faller DV. Platelet-derived growth factor (PDGF) induction of egr-1 is independent of PDGF receptor autophosphorylation on tyrosine. *J Biol Chem* 269: 16137–16142, 1994.

31. Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, Schweitzer R. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* 134: 2697–2708, 2007.

32. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 8: 221–233, 2007.

33. Perumal S, Antipova O, Orgel JPRO. Collagen fibril architecture, domain organization, and triple-helical conformation govern its proteolysis. *Proc Natl Acad Sci USA* 105: 2824–2829, 2008.

34. Poincloux R, Lizárraga F, Chavrier P. Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia. *Journal of Cell Science* 122: 3015–3024, 2009.

35. Poli M, Luscieti S, Gandini V, Maccarinelli F, Finazzi D, Silvestri L, Roetto A, Arosio P. Transferrin receptor 2 and HFE regulate furin expression via mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) signaling. Implications for transferrin-dependent hepcidin regulation. *Haematologica* 95: 1832–1840, 2010.

36. Popov C, Burggraf M, Kreja L, Ignatius A, Schieker M, Docheva D. Mechanical stimulation of human tendon stem/progenitor cells results in upregulation of matrix proteins, integrins and MMPs, and activation of p38 and ERK1/2 kinases. *BMC Mol Biol* 16: 6, 2015.

37. Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dünker N, Schweitzer R. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 136: 1351–1361, 2009.

38. Roberts WG, Whalen PM, Soderstrom E, Moraski G, Lyssikatos JP, Wang H-F, Cooper B, Baker DA, Savage D, Dalvie D, Atherton JA, Ralston S, Szewc R, Kath JC, Lin J, Soderstrom C, Tkalcevic G, Cohen BD, Pollack V, Barth W, Hungerford W, Ung E. Antiangiogenic and Antitumor Activity of a Selective PDGFR Tyrosine Kinase Inhibitor, CP-673,451. *Cancer Research* 65: 957–966, 2005.

39. Rolf CG, Fu BSC, Pau A, Wang W, Chan B. Increased cell proliferation and associated expression of PDGFRβ causing hypercellularity in patellar tendinosis. *Rheumatology (Oxford)* 40: 256–261, 2001.

40. Rutledge RG, Stewart D. Assessing the Performance Capabilities of LRE-Based Assays for Absolute Quantitative Real-Time PCR. *PLoS ONE* 5: e9731–11, 2010.

41. Sabeh F, Li X-Y, Saunders TL, Rowe RG, Weiss SJ. Secreted versus membrane-anchored collagenases: relative roles in fibroblast-dependent collagenolysis and invasion. *J Biol Chem* 284: 23001–23011, 2009.

42. Sabeh F. Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *The Journal of Cell Biology* 167: 769–781, 2004.

43. Schwartz AJ, Sarver DC, Sugg KB, Dzierzawski JT, Gumucio JP, Mendias CL. p38 MAPK signaling in postnatal tendon growth and remodeling. *PLoS ONE* 10: e0120044, 2015.

44. Scott A, Cook JL, Hart DA, Walker DC, Duronio V, Khan KM. Tenocyte responses to mechanical loading in vivo: a role for local insulin-like growth factor 1 signaling in early tendinosis in rats. *Arthritis Rheum* 56: 871–881, 2007.

45. Seiki M. Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. *Cancer Letters* 194: 1–11, 2003.

46. Sugg KB, Korn MA, Sarver DC, Markworth JF, Mendias CL. Inhibition of platelet-

derived growth factor signaling prevents muscle fiber growth during skeletal muscle hypertrophy. *FEBS Letters* (February 17, 2017). doi: 10.1002/1873-3468.12571.

47. Sugg KB, Lubardic J, Gumucio JP, Mendias CL. Changes in macrophage phenotype and induction of epithelial-to-mesenchymal transition genes following acute Achilles tenotomy and repair. *J Orthop Res* 32: 944–951, 2014.

48. Sun X, Gao X, Zhou L, Sun L, Lu C. PDGF-BB-induced MT1-MMP expression regulates proliferation and invasion of mesenchymal stem cells in 3-dimensional collagen via MEK/ERK1/2 and PI3K/AKT signaling. *Cellular Signalling* 25: 1279–1287, 2013.

49. Sweetwyne MT, Murphy-Ullrich JE. Thrombospondin1 in tissue repair and fibrosis: TGF-β-dependent and independent mechanisms. *Matrix Biology* 31: 178–186, 2012.

50. Tan T-W, Lai C-H, Huang C-Y, Yang W-H, Chen H-T, Hsu H-C, Fong Y-C, Tang C-H. CTGF enhances migration and MMP-13 up-regulation via αvβ3 integrin, FAK, ERK, and NF-κB-dependent pathway in human chondrosarcoma cells. *J Cell Biochem* 107: 345–356, 2009.

51. Taylor SH, Yeung C-YC, Kalson NS, Lu Y, Zigrino P, Starborg T, Warwood S, Holmes DF, Canty-Laird EG, Mauch C, Kadler KE. Matrix metalloproteinase 14 is required for fibrous tissue expansion. *eLife* 4: 1–38, 2015.

52. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* 3: 753–766, 2002.

53. Thomopoulos S, Das R, Sakiyama-Elbert S, Silva MJ, Charlton N, Gelberman RH. bFGF and PDGF-BB for Tendon Repair: Controlled Release and Biologic Activity by Tendon Fibroblasts In Vitro. *Ann Biomed Eng* 38: 225–234, 2009.

54. Thomopoulos S, Das R, Silva MJ, Sakiyama-Elbert S, Harwood FL, Zampiakis E, Kim HM, Amiel D, Gelberman RH. Enhanced flexor tendon healing through controlled delivery of PDGF-BB. *J Orthop Res* 27: 1209–1215, 2009.

55. Yang G, Rothrauff BB, Tuan RS. Tendon and ligament regeneration and repair: Clinical relevance and developmental paradigm. *Birth Defect Res C* 99: 203–222, 2013.

56. Yoshikawa Y, Abrahamsson S-O. Dose-related cellular effects of platelet-derived growth factor-BB differ in various types of rabbit tendons in vitro. *Acta Orthopaedica Scandinavica* 72: 287–292, 2009.

57. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 12: 21–35, 2010.

Chapter IV

Conclusions

Tendon is one of the primary effectors of locomotion by enabling efficient force transmission from skeletal muscle to bone. As with all biological systems, proper tendon function is highly dependent on the macromolecular structural organization and biochemical composition of its extracellular matrix (ECM). Tendon is a dynamic tissue, and as such, it reacts to externally applied loads by adapting its ECM, as evidenced by increases in tendon cross-sectional area (CSA), cell density, peak stress, peak strain and type I collagen content (19, 33-35, 42, 43, 67). While many of these adaptations are due to the ability of tendon fibroblasts to sense and respond to biomechanical cues in their local ECM environment (66, 69), the tendon ECM also selectively binds and releases various growth factors that regulate multiple cellular functions through the activation of transmembrane receptors and their downstream signaling cascades (3, 20). The objective of this dissertation was to determine the role of key signaling pathways activated during tendon growth and injury, and to investigate the biological mechanisms behind these responses. Since a central feature of tendon disorders is abnormal fibroblast morphology and a grossly disrupted appearance of the tendon ECM, my overall working hypothesis is molecular programs that regulate the interaction

between tendon fibroblasts and their local ECM environment during tendon growth play an important role in the healing response of injured tendons. Herein, we demonstrated that platelet-derived growth factor (PDGF) ligands and their receptors (PDGFRs) are necessary for postnatal tendon growth and remodeling in the adult. We utilized an integrative approach that coupled structural and functional studies of PDGFR-inhibited tendons with cell culture experiments that established the molecular mechanisms responsible for the observed differences in tendon growth. Taken together, this dissertation addresses fundamental gaps in the field of musculoskeletal biology by providing a focused examination of key molecular programs activated in response to tendon injury, namely epithelial-to-mesenchymal transition (EMT) and matrix metalloproteinase (MMP) genes (Chapter II). Using an informative overload model that recapitulates the critical elements of tendon growth, we found that PDGFR signaling regulates changes in tendon structural and functional properties in response to mechanical growth stimuli (Chapter III). The main conclusions of this dissertation are briefly summarized as follows:

1. M2 macrophages are normally present within the interfascicular matrix or endotenon of uninjured tendons. Tendon transection followed by acute repair leads to an early and dramatic influx of M1 macrophages within the injured tendon tissue. M2 macrophages slowly accumulate in areas of newly formed tendon tissue and become the predominant macrophage phenotype by 28 days (Chapter II).

2. Tendon transection followed by acute repair is accompanied by significant changes in the tendon transcriptome, including activation of genes in the EMT and MMP families (Chapter II).
3. Tendon fibroblasts express both PDGFRα and PDGFRβ, and signaling through these receptors is required for postnatal tendon growth and remodeling in the adult, primarily due to defects in proliferation and migration of tendon fibroblasts (Chapter III).
4. Membrane type-1 matrix metalloproteinase (MT1-MMP) is an essential proteinase for tendon fibroblast migration through cross-linked type I collagen hydrogels that closely recapitulate the tendon ECM environment in vivo (Chapter III).
5. PDGF-BB-dependent MT1-MMP expression by tendon fibroblasts is regulated via PI3K/Akt-mediated translational and ERK1/2-mediated post-translational mechanisms. Inhibition of PI3K/Akt and ERK1/2 pathways also prevents PDGF-BB-dependent membrane trafficking of active MT1-MMP to the cell surface in tendon fibroblasts (Chapter III).

In recent years, the most significant advances in tendon research that have contributed to our current understanding of tendon biology have occurred in the field of tendon development (23, 26, 29, 45, 51, 52). The motivation for many of these developmental studies in tendon stems from the clinically relevant problem of tendinopathy. In this regard, findings from studies of tendon development have made a substantial impact on research efforts aimed at understanding the molecular biology of

tendon injury and repair, by facilitating the generation of *ScxGFP* reporter mice, as well as constitutive and inducible Cre lines driven by the Scx promoter, to be used as experimental tools (9, 51). Several transcription factors with functional roles in tendon differentiation, including Scx (45), mohawk (Mkx) (29), and early growth response 1 and 2 (Egr1 and Egr2) (17, 38), are also known to be upregulated following tendon injury suggesting that these transcription factors may play dual roles in tendon development and regeneration (Chapter II) (13, 59). Additionally, the transcription factors Mkx and Egr1 have been used to promote tenogenesis of mesenchymal stem cells for implantation in critical tendon defects to encourage healing in various animal models (17, 41). Similar to developmental studies of tendon, fully elucidating the molecular mechanisms that govern tendon growth and remodeling may also inform regenerative strategies for the treatment of tendon injuries and diseases.

Commentary and Future Directions

The importance of macrophages to the healing of injured musculoskeletal tissues is well documented (64). In Chapter II, we demonstrated the precise location of M1 and M2 macrophages in uninjured tendon tissue, as well as the sequence and timing of their accumulation following a physiologically relevant tendon injury. Compared to skeletal muscle tissue, M1 macrophages persist longer and M2 macrophages appear later. This may partially explain the protracted course of tendon healing relative to skeletal muscle tissue. It may also offer an opportunity to accelerate tissue healing in tendons after an injury occurs, either by promoting earlier removal of the M1 macrophages or the earlier

arrival of M2 macrophages. However, a major limitation to macrophage research is the absence of transgenic animals models that can target specific macrophage subpopulations (47). At the moment, transient reductions in entire macrophage populations is possible via antibody-mediated depletion by targeting the colony stimulating factor 1 receptor (CSF1R) (11), diphtheria-toxin mediated killing (54) or administration of gadolinium chloride or clodronate liposomes (2, 15). However, alternative approaches would be to use a drug such as azithromycin or fasudil (40, 46) that induces an earlier switch in macrophage phenotype from M1 to M2, or cell-based therapies where M2 macrophages are isolated and injected into the site of repair. This technique has demonstrated mixed results in the past when used in diabetic cutaneous wounds or traumatic muscle injury (30, 48), but has not been tried in injured tendon tissue, and perhaps the results are tissue dependent. Taken together, more sophisticated transgenic animal models are greatly needed that can selectively delete M1 and M2 macrophages with spatiotemporal control, and therefore more accurately address the molecular mechanisms responsible for impaired tendon regeneration.

Using a physiologically relevant tendon injury model (Chapter II), we identified that Snail1 and other similar genes that play important roles in EMT-dependent processes were coordinately regulated throughout the tendon repair process. These EMT-related genes not only play a central role in the EMT program, but are also involved in well established EMT-independent functions, including ECM remodeling, cell lineage specification and cell cycle regulation (6, 56, 65). While whole tendons can be dissociated to release putative tendon stem cells that have higher proliferative rates than tendon fibroblasts and possess the ability to differentiate into multiple other cell

types (8), the location and identity of these cells in vivo within intact tendons remains unknown. Several lines of evidence suggest that these cells might be concentrated in the epitenon (19, 43, 55, 60), which is a loose epithelial-like tissue layer that surrounds the tendon and is subtended by a basement membrane, a specialized form of ECM that is normally found beneath all epithelial cells. In other tissues, the epithelium can potentially serve as a source of fibroblasts during responses to tissue injury through a process known as EMT (31). However, in the case of tendon, whether the fibroblasts emerging from the epitenon in response to mechanical loading are of epithelial origin remains unknown. To address this question, a marker specific of the epitenon would need to be identified such that an inducible Cre driver could be made, and then crossed to a *Rosa26-stopf/f-tdTomato* mouse that is in the *ScxGFP* background (Figure 4.1). Using this approach, a lineage tracing experiment could be performed using a murine model of intrasynovial flexor tendon repair of the flexor digitorum longus (FDL) tendon (22). Not all tendons in the mouse have a well developed epitenon, especially the Achilles and plantaris tendons, but the tail tendons and flexor tendons of the fore and hind paws do have a basement membrane-like structure that stains positive for laminin and type IV collagen (unpublished data). Prior to acute transection and repair of the FDL tendon, we could induce permanent expression of tdTomato in all epithelial cells following tamoxifen treatment, and identify whether the fibroblasts that accumulate in the injured tendons are derived from epithelial cells. The presence of yellow cells (red and green overlap) would indicate fibroblasts that arose from epithelial progenitors, while fibroblasts that appeared green would indicate that EMT is not likely involved in tendon regeneration.

Other studies described in this dissertation (Chapter III) establish the importance of PDGFR signaling in tendon growth and remodeling in the adult. Our findings also provide compelling evidence for the role of at least one PDGF isoform in the proliferation and migration of tendon fibroblasts. However, a major obstacle encountered by studies of PDGF function is that signaling through PDGFRα and PDGFRβ in vitro demonstrates functional redundancy, while these receptors have distinct roles and expression patterns in vivo (68). One limitation of the studies described in this dissertation is that the use of a PDGFR inhibitor in vivo results in a global reduction of PDGFR signaling within tendon due to pharmacological blockade of both PDGFRα and PDGFRβ. Therefore, it is not possible to determine which PDGF isoforms and PDGFR subtypes are responsible for the proliferation and migration of tendon fibroblasts in the current studies. To determine the functional importance of the PDGFR subtypes in vivo, the following experiments are proposed. Since deletion of PDGFRα and PDGFRβ results in early embryonic lethality (25), spatiotemporal control of PDGFRα and PDGFRβ inactivation is required. To accomplish this, three mouse models (*PDGFRαKD*, *PDGFRβKD* and *PDGFRαβKD*) could be generated for the conditional knockdown of either PDGFRα, PDGFRβ or both PDGFRα and PDGFRβ specifically in tendon fibroblasts by crossing a $Scx^{CreERT2/+}$ knock-in mouse with $PDGFR\alpha^{f/f}$, $PDGFR\beta^{f/f}$ and $PDGFR\alpha^{f/f}$:$PDGFR\beta^{f/f}$, respectively (Figure 4.2). The $Scx^{CreERT2/+}$ mice also have the ScxGFP transgene (51), which allows for the convenient identification of scleraxis (Scx)-expressing cells. In the *PDGFRαKD*, *PDGFRβKD* and *PDGFRαβKD*, CreERT2 is driven by the tendon-specific Scx promoter under the inducible control of tamoxifen. Mice would receive daily systemic injections of tamoxifen

for 3 days prior to overload and throughout the remainder of the study period. *PDGFR$\alpha^{f/f}$*, *PDGFR$\beta^{f/f}$* and *PDGFR$\alpha^{f/f}$:PDGFR$\beta^{f/f}$* mice do not have the CreERT2 allele, and thus will also receive tamoxifen treatment to serve as wild-type controls (*PDGFRαWT*, *PDGFRβWT* and *PDGFR$\alpha\beta$WT*). Hindlimb synergist ablation will be performed as described in Chapter III and the mice will be allowed to recover for 3 or 10 days, at which time the left plantaris tendons will be collected for gene expression analysis, and the right plantaris tendons will be used for histological examination or mechanical properties measurements. Efficiency of knockdown will be determined using qPCR with genomic DNA and primers that can quantify the floxed and null alleles. Collectively, the above experiments will help elucidate the specific functions of the PDGFR subtypes during postnatal tendon growth and remodeling in vivo.

While inhibition of PDGFR signaling suppressed the normal growth of tendon tissue after mechanical overload (Chapter III), we had previously shown that PDGFR signaling is also required for skeletal muscle hypertrophy (58). This raises an important question of whether the consequences of PDGFR inhibition on tendon growth are direct or indirect, or in other words, is tendon growth possible in the absence of muscle hypertrophy? The primary function of tendon is to transmit load, and typically this load is generated proximally by muscular contraction. Muscle hypertrophy leads to increased force production, which increases the load transmitted by tendon, and this consequently results in tendon growth. However, tendons can also transmit loads that originate distally, such as those that arise during the heel strike phase of the gait cycle, and these loads are independent of muscular contraction. Thus, it remains possible that tendon growth can occur in the absence of muscle hypertrophy, although probably to a lesser

extent. To clarify this issue, experiments could be performed that genetically inactivate PDGFR signaling in a tissue specific manner using transgenic mouse models. Furthermore, analysis of the gene expression data sets from PDGFR-inhibited skeletal muscle and tendon tissue demonstrates overlapping changes in MMP expression, particularly with regard to MT1-MMP expression. This further highlights the significance of the complementary in vitro work in Chapter III identifying MT1-MMP as an essential proteinase for tendon fibroblast migration through tendon ECM, and provides additional support for the direct effect of PDGFR inhibition on tendon growth.

Compared to other receptor tyrosine kinases (RTKs), there is less evidence to support the role of PDGFR signaling in many normal physiological functions such as tissue growth in the adult, despite its importance during embryonic development and in numerous pathological conditions (3). On the other hand, insulin-like growth factor 1 (IGF-1) is another member of the RTK family that has been implicated in the growth of musculoskeletal tissues, and has been shown to be a positive regulator of skeletal muscle mass through its binding to the insulin-like growth factor 1 receptor (IGF1R) (1, 18). Similar to the studies proposed for PDGFR signaling in this dissertation, it would be very interesting to determine the role of IGF1R signaling in postnatal tendon growth and remodeling. At the whole tissue level, IGF-1 appears to play an important role in type I collagen synthesis. Exercise and physical training can lead to increases in the local expression of IGF-1 in skeletal muscle and tendon tissue, accompanied by increases in type I collagen synthesis (24, 37, 44). Furthermore, local administration of IGF-1 in a collagenase model of flexor tendinitis in horses led to a reduction in core lesion size compared to saline injection, along with increases in tendon collagen content and

tendon stiffness (10). In a recent human study, injection of IGF-1 into the patellar tendon of healthy adults stimulated collagen synthesis both within the tendon as well as in the peritendinous tissue (21). In a rat overuse injury model, tendinopathic changes in the supraspinatus tendon were accompanied by reduced expression of type I and III collagen, IGF-1 and other genes associated with protein synthesis (5).

While the signaling pathways activated by IGF-1 have not been described in detail in tendon, IGF-1 is known to have a potent effect on activating the phosphoinositide 3-kinase (PI3K) pathway with subsequent activation of p70 S6 kinase (p70S6K) via mechanistic target of rapamycin complex 1 (mTORC1), which increases ribosomal biogenesis and initiates mRNA translation (18). However, it remains to be determined whether the increases in type I collagen synthesis being measured at the whole tissue level following local IGF-1 administration are a direct effect of IGF-1 binding to IGF1R, or are being mediated through another cell type, perhaps through the production of an intermediary factor or other signaling molecule. Additionally, IGF-1 has been shown to regulate the expression of other matrix proteins such as proteoglycans (PGs) and glycosoaminoglycans (GAGs) such as hyaluronic acid (28, 36, 61). Hyaluronic acid is responsible for forming an immature matrix during tendon growth that can serve as a scaffold for migrating cells and is later replaced by mature type I collagen (55). Thus, in addition to the regulation of type I collagen synthesis, another potential mechanism of IGF1R signaling during tendon growth is the imitation of matrix formation through hyaluronic acid production.

To determine the role of IGF1R signaling during tendon growth and remodeling in the adult, the following experiments are proposed. First, cultured tendon fibroblasts

could be treated with a time course of IGF-1 to determine which major downstream signaling pathways are activated, and to determine if there is an IGF-1-dependent increase in type I collagen mRNA or protein levels, as well as the hyaluronic acid synthase enzymes. Small molecule inhibitors could then be used to block specific signaling pathways that were activated downstream of the IGF1R to measure their effect on matrix production. Next, to test the requirement of IGF1R signaling for tendon growth in vivo, *ScxCreERT2* mice will be crossed to *IGF1R*$^{f/f}$ mice and subjected to mechanical overload via the hindlimb synergist ablation model. Upon tamoxifen treatment, the IGF1R will be knocked down in Scx-expressing cells. If IGF1R signaling is regulating matrix production during tendon growth, we would expect a decrease in tendon CSA in the absence of IGF1R expression on tendon fibroblasts. Follow-up studies with proteomics, histology and electron microscopy will help provide an idea of the composition of the matrix in the mutant animals. Tendon mechanics can also be performed to test tendon function. These studies would provide mechanistic insights into the regulation of tendon ECM production with potential clues about the timing of therapy to encourage new matrix production in chronically degenerated tendons.

Our findings in this dissertation earmark the growth factor PDGF-BB and proteinase MT1-MMP as potential targets for therapeutic intervention. Indeed, translational studies have already utilized the controlled delivery of PDGF-BB to enhance tendon healing in a canine model of intrasynovial flexor tendon repair with modest success (16, 62, 63). In Chapter III, our results demonstrate that PDGFR signaling primarily regulates proliferation and migration of tendon fibroblasts rather than ECM production, which may help better inform the timing of delivery of this growth factor

when used to augment tendon healing following injury. Recombinant human PDGF-BB has also been used to promote cutaneous wound healing in various diabetic animal models with mixed results (32, 39, 49, 50). However, PDGF-BB demonstrated encouraging results in a collagenase model of Achilles tendinopathy in a rat by improving mechanical properties compared to other commonly used treatments, including platelet-rich plasma (PRP) and steroids (57). Given that our findings demonstrate that PDGF-BB treatment of cultured tendon fibroblasts increases their proliferation and migration, logically it follows that intratendinous injections of recombinant human PDGF-BB into regions of hypocellularity and degenerated ECM in tendinopathic tendons may lead to the recruitment of local tendon fibroblasts and eventual tissue healing. Compared to PRP injections that contain variable amounts of PDGF-BB as well as numerous other growth factors (27), a potential advantage of purified recombinant human PDGF-BB is reproducible results due to precise dosing that also avoids the confounding effects of other growth factors present in PRP formulations.

Finally, targeting MT1-MMP may also hold promise in the treatment of painful tendinopathies, since dysregulation of MMP activity is thought to be one of the underlying causes (12). In addition to degradation of multiple ECM components, other functions of MT1-MMP include activation of other secreted MMPs such as MMP2 (7), cleavage of matrix proteins that can create protein fragments then serve as signaling molecules or exposing cryptic epitopes that control angiogenesis in the tendon ECM that can lead to the infiltration of inflammatory cells (12, 53). There is also evidence to suggest that MT1-MMP can lead to the upregulation of COX-2, which is a potent mediator of pain and inflammation(4, 12) Low-level dosing of doxycycline, a potent

MMP inhibitor that also has antimicrobial properties, may block the activation of MMPs by MT1-MMP, although further clinical studies are needed before any definitive conclusions can be made (14). Taken together, our results from Chapters II and III suggest that MMP inhibition needs to be approached with caution because while inhibition may be helpful in pain and inflammation, it may also attenuate endogenous growth factor stimulated tendon fibroblast recruitment, which may in turn impair healing of the injured tendon.

In summary, studies in this dissertation contribute to a growing body of work in the field of musculoskeletal regenerative medicine, and as our understanding of the cellular and molecular mechanisms of tendon biology advances, we will continue to improve therapies for the management of tendon injuries and diseases.

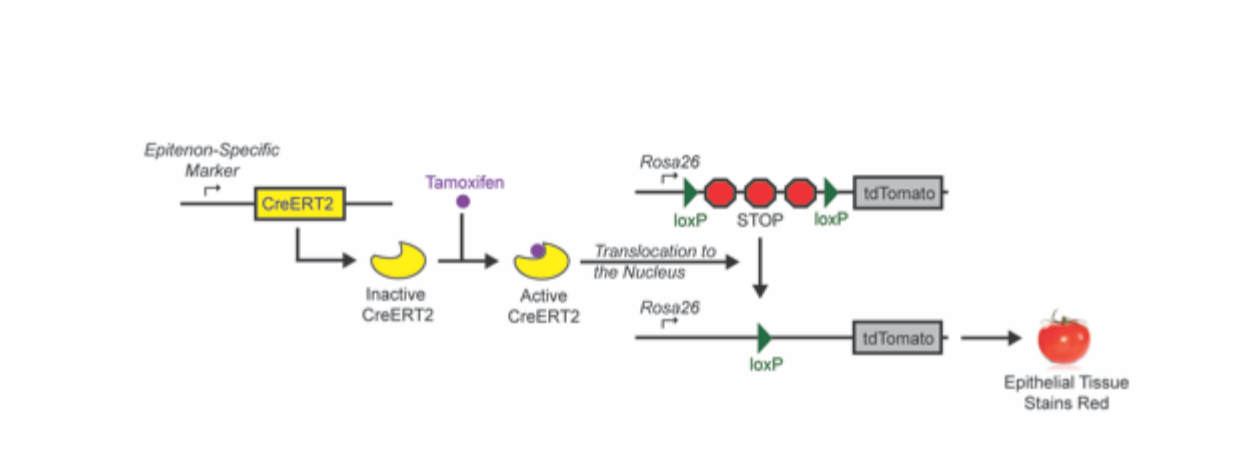

Figure 4.1. Targeting scheme for lineage tracing experiments.

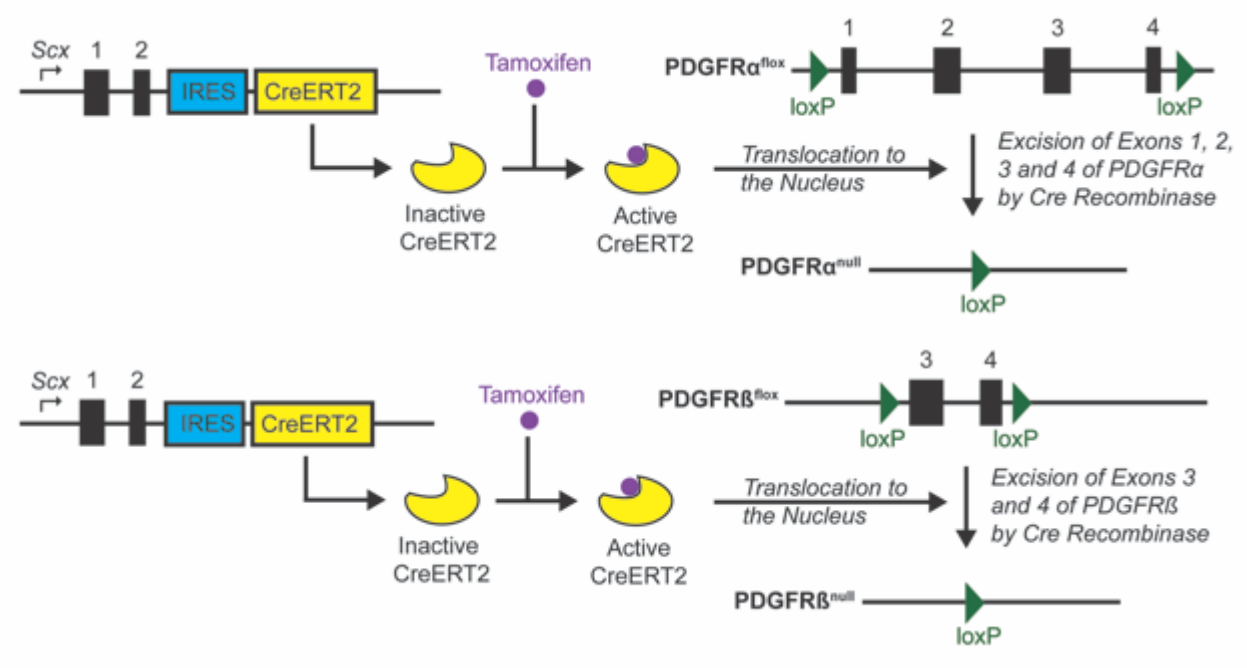

Figure 4.2. Targeting scheme for genetic inactivation of PDGFRα and PDGFRβ.

References

1. Adams GR. Invited Review: Autocrine/paracrine IGF-I and skeletal muscle adaptation. *Journal of Applied Physiology* 93: 1159–1167, 2002.

2. Ahmad N, Gardner CR, Yurkow EJ, Laskin DL. Inhibition of macrophages with gadolinium chloride alters intercellular adhesion molecule-1 expression in the liver during acute endotoxemia in rats. *Hepatology* 29: 728–736, 1999.

3. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes & Development* 22: 1276–1312, 2008.

4. Annabi B, Laflamme C, Sina A, Lachambre M-P, Béliveau R. A MT1-MMP/NF-κB signaling axis as a checkpoint controller of COX-2 expression in CD133(+) U87 glioblastoma cells. *J Neuroinflammation* 6: 8–10, 2009.

5. Archambault JM, Jelinsky SA, Lake SP, Hill AA, Glaser DL, Soslowsky LJ. Rat supraspinatus tendon expresses cartilage markers with overuse. *J Orthop Res* 25: 617–624, 2007.

6. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132: 3151–3161, 2005.

7. Bernardo MM, Fridman R. TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type 1)-MMP. *Biochem J* 374: 739–745, 2003.

8. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, Li L, Leet AI, Seo B-M, Zhang L, Shi S, Young MF. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 13: 1219–1227, 2007.

9. Blitz E, Sharir A, Akiyama H, Zelzer E. Tendon-bone attachment unit is formed modularly by a distinct pool of Scx- and Sox9-positive progenitors. *Development* 140: 2680–2690, 2013.

10. Dahlgren LA, van der Meulen MCH, Bertram JEA, Starrak GS, Nixon AJ. Insulin-like growth factor-I improves cellular and molecular aspects of healing in a collagenase-induced model of flexor tendinitis. *J Orthop Res* 20: 910–919, 2002.

11. Dai X-M, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, Sylvestre V, Stanley ER. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* 99: 111–120, 2002.

12. Del Buono A, Oliva F, Osti L, MAFFULLI N. Metalloproteases and tendinopathy. *Muscles Ligaments Tendons J* 3: 51–57, 2013.

13. Dyment NA, Liu C-F, Kazemi N, Aschbacher-Smith LE, Kenter K, Breidenbach AP, Shearn JT, Wylie C, Rowe DW, Butler DL. The Paratenon Contributes to Scleraxis-Expressing Cells during Patellar Tendon Healing. *PLoS ONE* 8: e59944, 2013.

14. Fallon K, Purdam C, Cook J, Lovell G. A “polypill” for acute tendon pain in athletes with tendinopathy? *Journal of Science and Medicine in Sport* 11: 235–238, 2008.

15. Ferenbach DA, Sheldrake TA, Dhaliwal K, Kipari TMJ, Marson LP, Kluth DC, Hughes J. Macrophage/monocyte depletion by clodronate, but not diphtheria toxin, improves renal ischemia/reperfusion injury in mice. *Kidney Int* 82: 928–933, 2012.

16. Gelberman RH, Thomopoulos S, Sakiyama-Elbert SE, Das R, Silva MJ. The early effects of sustained platelet-derived growth factor administration on the functional and structural properties of repaired intrasynovial flexor tendons: an in vivo biomechanic study at 3 weeks in canines. *J Hand Surg Am* 32: 373–379, 2007.

17. Guerquin M-J, Charvet B, Nourissat G, Havis E, Ronsin O, Bonnin M-A, Ruggiu M, Olivera-Martinez I, Robert N, Lu Y, Kadler KE, Baumberger T, Doursounian L, Berenbaum F, Duprez D. Transcription factor EGR1 directs tendon differentiation and promotes tendon repair. *J Clin Invest* 123: 3564–3576, 2013.

18. Gumucio JP, Mendias CL. Atrogin-1, MuRF-1, and sarcopenia. *Endocrine* 43: 12–21, 2012.

19. Gumucio JP, Phan AC, Ruehlmann DG, Noah AC, Mendias CL. Synergist ablation induces rapid tendon growth through the synthesis of a neotendon matrix. *J Appl Physiol* 117: 1287–1291, 2014.

20. Gumucio JP, Sugg KB, Mendias CL. TGF-β Superfamily Signaling in Muscle and Tendon Adaptation to Resistance Exercise. *Exercise and Sport Sciences Reviews* 43: 93–99, 2015.

21. Hansen M, Boesen A, Holm L, Flyvbjerg A, Langberg H, KJAeR M. Local administration of insulin-like growth factor-I (IGF-I) stimulates tendon collagen synthesis in humans. *Scandinavian Journal of Medicine & Science in Sports* (January 31, 2012). doi: 10.1111/j.1600-0838.2011.01431.x.

22. Hasslund S, O’Keefe RJ, Awad HA. A Mouse Model of Flexor Tendon Repair. In: *Skeletal Development and Repair*. Totowa, NJ: Humana Press, 2014, p. 73–88.

23. Havis E, Bonnin M-A, Olivera-Martinez I, Nazaret N, Ruggiu M, Weibel J, Durand C, Guerquin M-J, Bonod-Bidaud C, Ruggiero F, Schweitzer R, Duprez D.

Transcriptomic analysis of mouse limb tendon cells during development. *Development* 141: 3683–3696, 2014.

24. Heinemeier KM, Mackey AL, Doessing S, Hansen M, Bayer ML, Nielsen RH, Herchenhan A, Malmgaard-Clausen NM, KJAeR M. GH/IGF-I axis and matrix adaptation of the musculotendinous tissue to exercise in humans. *Scandinavian Journal of Medicine & Science in Sports* 22: e1–e7, 2012.

25. Hoch RV. Roles of PDGF in animal development. *Development* 130: 4769–4784, 2003.

26. Huang AH, Riordan TJ, Pryce B, Weibel JL, Watson SS, Long F, Lefebvre V, Harfe BD, Stadler HS, Akiyama H, Tufa SF, Keene DR, Schweitzer R. Musculoskeletal integration at the wrist underlies the modular development of limb tendons. *Development* 142: 2431–2441, 2015.

27. Hudgens JL, Sugg KB, Grekin JA, Gumucio JP, Bedi A, Mendias CL. Platelet-Rich Plasma Activates Proinflammatory Signaling Pathways and Induces Oxidative Stress in Tendon Fibroblasts. *Am J Sports Med* 44: 1931–1940, 2016.

28. Imai Y, Odajima R, Inoue Y, Shishiba Y. Effect of growth factors on hyaluronan and proteoglycan synthesis by retroocular tissue fibroblasts of Graves' ophthalmopathy in culture. *Acta Endocrinol* 126: 541–552, 1992.

29. Ito Y, Toriuchi N, Yoshitaka T, Ueno-Kudoh H, Sato T, Yokoyama S, Nishida K, Akimoto T, Takahashi M, Miyaki S, Asahara H. The Mohawk homeobox gene is a critical regulator of tendon differentiation. *Proc Natl Acad Sci USA* 107: 10538–10542, 2010.

30. Jetten N, Roumans N, Gijbels MJ, Romano A, Post MJ, de Winther MPJ, van der Hulst RRWJ, Xanthoulea S. Wound Administration of M2-Polarized Macrophages Does Not Improve Murine Cutaneous Healing Responses. *PLoS ONE* 9: e102994–9, 2014.

31. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 119: 1420–1428, 2009.

32. Keshava R, Gope R. Sodium Butyrate Plus EGF and PDGF-BB Aids Cutaneous Wound Healing in Diabetic Mice. *Advances in Biology* 2015: 1–10, 2015.

33. KJAeR M, Langberg H, Heinemeier K, Bayer ML, Hansen M, Holm L, Doessing S, Kongsgaard M, Krogsgaard MR, Magnusson SP. From mechanical loading to collagen synthesis, structural changes and function in human tendon. *Scandinavian Journal of Medicine & Science in Sports* 19: 500–510, 2009.

34. Kjaer M, Magnusson P, Krogsgaard M, Moller JB, Olesen J, Heinemeier K, Hansen M, Haraldsson B, Koskinen S, Esmarck B, Langberg H. Extracellular matrix adaptation of tendon and skeletal muscle to exercise. *J Anat* 208: 445–

450, 2006.

35. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiological Reviews* 84: 649–698, 2004.

36. Kuroda K, Utani A, Hamasaki Y, Shinkai H. Up-regulation of putative hyaluronan synthase mRNA by basic fibroblast growth factor and insulin-like growth factor-1 in human skin fibroblasts. *J Dermatol Sci* 26: 156–160, 2001.

37. Langberg H, Skovgaard D, Petersen LJ, Bulow J, KJAeR M. Type I collagen synthesis and degradation in peritendinous tissue after exercise determined by microdialysis in humans. *The Journal of Physiology* 521 Pt 1: 299–306, 1999.

38. Léjard V, Blais F, Guerquin M-J, Bonnet A, Bonnin M-A, Havis E, Malbouyres M, Bidaud CB, Maro G, Gilardi-Hebenstreit P, Rossert J, Ruggiero F, Duprez D. EGR1 and EGR2 Involvement in Vertebrate Tendon Differentiation. *J Biol Chem* 286: 5855–5867, 2011.

39. Li H, Fu X, Zhang L, Huang Q, Wu Z, Sun T. Research of PDGF-BB Gel on the Wound Healing of Diabetic Rats and Its Pharmacodynamics. *Journal of Surgical Research* 145: 41–48, 2008.

40. Liu C, Li Y, Yu J, Feng L, Hou S, Liu Y, Guo M, Xie Y, Meng J, Zhang H, Xiao B, Ma C. Targeting the Shift from M1 to M2 Macrophages in Experimental Autoimmune Encephalomyelitis Mice Treated with Fasudil. *PLoS ONE* 8: e54841–13, 2013.

41. Liu H, Zhang C, Zhu S, Lu P, Zhu T, Gong X, Zhang Z, Hu J, Yin Z, Heng BC, Chen X, Wei Ouyang H. Mohawk Promotes the Tenogenesis of Mesenchymal Stem Cells Through Activation of the TGFβ Signaling Pathway. *STEM CELLS* 33: 443–455, 2015.

42. Magnusson SP, Hansen P, KJAeR M. Tendon properties in relation to muscular activity and physical training. *Scandinavian Journal of Medicine & Science in Sports* 13: 211–223, 2003.

43. Mendias CL, Gumucio JP, Bakhurin KI, Lynch EB, Brooks SV. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. *J Orthop Res* 30: 606–612, 2011.

44. Miller BF, Olesen JL, Hansen M, Døssing S, Crameri RM, Welling RJ, Langberg H, Flyvbjerg A, Kjaer M, Babraj JA, Smith K, Rennie MJ. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *The Journal of Physiology* 567: 1021–1033, 2005.

45. Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, Schweitzer R. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* 134: 2697–

2708, 2007.

46. Murphy BS, Sundareshan V, Cory TJ, Hayes D, Anstead MI, Feola DJ. Azithromycin alters macrophage phenotype. *Journal of Antimicrobial Chemotherapy* 61: 554–560, 2008.

47. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol* 89: 557–563, 2011.

48. Novak ML, Weinheimer-Haus EM, Koh TJ. Macrophage activation and skeletal muscle healing following traumatic injury. *J Pathol* 232: 344–355, 2014.

49. Park SA, Raghunathan VK, Shah NM, Teixeira L, Motta MJ, Covert J, Dubielzig R, Schurr M, Isseroff RR, Abbott NL, McAnulty J, Murphy CJ. PDGF-BB Does Not Accelerate Healing in Diabetic Mice with Splinted Skin Wounds. *PLoS ONE* 9: e104447–8, 2014.

50. Pierce GF, Mustoe TA, Altrock BW, Deuel TF, Thomason A. Role of platelet-derived growth factor in wound healing. *J Cell Biochem* 45: 319–326, 1991.

51. Pryce BA, Brent AE, Murchison ND, Tabin CJ, Schweitzer R. Generation of transgenic tendon reporters, ScxGFP and ScxAP, using regulatory elements of the scleraxis gene. *Dev Dyn* 236: 1677–1682, 2007.

52. Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dünker N, Schweitzer R. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 136: 1351–1361, 2009.

53. Riley G. The pathogenesis of tendinopathy. A molecular perspective. *Rheumatology (Oxford)* 43: 131–142, 2004.

54. Saxena V, Ondr JK, Magnusen AF, Munn DH, Katz JD. The Countervailing Actions of Myeloid and Plasmacytoid Dendritic Cells Control Autoimmune Diabetes in the Nonobese Diabetic Mouse. *J Immunol* 179: 5041–5053, 2007.

55. Schwartz AJ, Sarver DC, Sugg KB, Dzierzawski JT, Gumucio JP, Mendias CL. p38 MAPK signaling in postnatal tendon growth and remodeling. *PLoS ONE* 10: e0120044, 2015.

56. Sekiya SS, Suzuki AA. Glycogen synthase kinase 3 β-dependent Snail degradation directs hepatocyte proliferation in normal liver regeneration. *Proc Natl Acad Sci USA* 108: 11175–11180, 2011.

57. Shah V, Bendele A, Dines JS, Kestler HK, Hollinger JO, Chahine NO, Hee CK. Dose-response effect of an intra-tendon application of recombinant human platelet-derived growth factor-BB (rhPDGF-BB) in a rat Achilles tendinopathy model. *J Orthop Res* 31: 413–420, 2012.

58. Sugg KB, Korn MA, Sarver DC, Markworth JF, Mendias CL. Inhibition of platelet-derived growth factor signaling prevents muscle fiber growth during skeletal muscle hypertrophy. *FEBS Letters* (February 17, 2017). doi: 10.1002/1873-3468.12571.

59. Sugg KB, Lubardic J, Gumucio JP, Mendias CL. Changes in macrophage phenotype and induction of epithelial-to-mesenchymal transition genes following acute Achilles tenotomy and repair. *J Orthop Res* 32: 944–951, 2014.

60. Taylor SH, Al-Youha S, Van Agtmael T, Lu Y, Wong J, McGrouther DA, Kadler KE. Tendon Is Covered by a Basement Membrane Epithelium That Is Required for Cell Retention and the Prevention of Adhesion Formation. *PLoS ONE* 6: e16337, 2011.

61. Thiébot B, Bichoualne L, Langris M, Bonnamy PJ, Barbey P, Carreau S, Bocquet J. IGF-1 stimulates synthesis of undersulfated proteoglycans and of hyaluronic acid by peritubular cells from immature rat testis. *Biochim Biophys Acta* 1358: 127–141, 1997.

62. Thomopoulos S, Das R, Silva MJ, Sakiyama-Elbert S, Harwood FL, Zampiakis E, Kim HM, Amiel D, Gelberman RH. Enhanced flexor tendon healing through controlled delivery of PDGF-BB. *J Orthop Res* 27: 1209–1215, 2009.

63. Thomopoulos S, Zaegel M, Das R, Harwood FL, Silva MJ, Amiel D, Sakiyama-Elbert S, Gelberman RH. PDGF-BB released in tendon repair using a novel delivery system promotes cell proliferation and collagen remodeling. *J Orthop Res* 25: 1358–1368, 2007.

64. Tidball JG. Inflammatory processes in muscle injury and repair. *AJP: Regulatory, Integrative and Comparative Physiology* 288: R345–R353, 2004.

65. Vega S. Snail blocks the cell cycle and confers resistance to cell death. *Genes & Development* 18: 1131–1143, 2004.

66. Wang N, Tytell JD, Ingber DE. Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. *Nat Rev Mol Cell Biol* 10: 75–82, 2009.

67. Woo SL, Ritter MA, Amiel D, Sanders TM, Gomez MA, Kuei SC, Garfin SR, Akeson WH. The biomechanical and biochemical properties of swine tendons--long term effects of exercise on the digital extensors. *Connect Tissue Res* 7: 177–183, 1980.

68. Wu E, Palmer N, Tian Z, Moseman AP, Galdzicki M, Wang X, Berger B, Zhang H, Kohane IS. Comprehensive Dissection of PDGF-PDGFR Signaling Pathways in PDGFR Genetically Defined Cells. *PLoS ONE* 3: e3794, 2008.

69. Yu H, Mouw JK, Weaver VM. Forcing form and function: biomechanical regulation

of tumor evolution. *Trends in Cell Biology* 21: 47–56, 2011.